

**School of Biomedical Sciences**

**The role of bone morphogenetic proteins (BMPs) in ovarian function**

**Sarmed Al-Samerria**

**This thesis is presented for the Degree of  
Doctor of Philosophy  
Curtin University**

**September 2016**

## Declaration

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*To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.*

*This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.*

*The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the University of New England Animal Ethics Committee.*



**Sarmed Al-Samerria**  
**1<sup>st</sup> of August 2016**

## Abstract

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This study is conducted to increase the understanding of ovarian follicle development in order to enhance and preserve female fertility in mammalian species including experimental animals such as mice, production animals such as sheep and is ultimately applicable in humans. The primordial follicles, their number and quality determine the longevity and quality of female fertility. Regulation of the primordial to the primary follicle transition is considered an important factor in preventing depletion of female fertility reserve. Ovarian follicle development has mainly thought to be regulated by the action of gonadotropins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH). However, recent studies, generated in our laboratory, and that of others, have found that the initial recruitment of primordial follicles is in fact, independent of gonadotropin hormones and is suggested to be controlled by various intra-ovarian factors, among which are bone morphogenetic proteins (BMP).

It was first thought that BMPs were involved indirectly, through regulating gonadotropin production in the pituitary glands. However, some other reports indicate otherwise and suggest that ovarian follicle development process is more complicated and regulated by the action and interaction between BMP signalling and gonadotropin hormones. However, the mechanism of action, interaction and at which level the interactions occur (intracellular or extracellular) was not clearly understood. Therefore, with the overall objectives of elucidating the role of BMPs, the specific aims of this study were:

- (I) Investigation of the temporal-spatial protein expression of the BMP receptor 1B (BMPR-1B), follicle stimulating hormone (FSHR) and luteinising hormone receptor (LHR) during 4 stages of follicle development in sheep.
- (II) Study of the role of BMP signalling in the regulation of folliculogenesis including the early stage of follicle development, and its influences on primary follicle survival rate and ovulation, using mice passively immunised against BMP receptor 1B (BMPR-1B) and BMP-4 ligand.
- (III) Exploration of the mechanism of interaction between the gonadotropin and BMP signalling during follicle development by examining the impact of passive immunisation on the protein receptor expression of BMPR-1B, FSHR and LHR,

using immunofluorescent localisation combined with 3D image analysis to quantify the intensity of the receptors' protein expression.

- (IV) Investigation of the interaction between BMP signalling and gonadotropins (FSHR and LHR) at the transcript level (mRNA).

As part of the first objective of this thesis and to examine the temporal-spatial protein expression of the BMP receptor BMPR-1B, FSHR and LHR during different stages of follicle development, the expression of these receptors was analysed *in situ* and determined quantitatively using computerized 3D-image analysis. The result of this study showed that BMPR-1B was expressed in the granulosa cells at all stages of follicle development, while FSHR was detected in granulosa cells of the primary follicles onward and LHR was absent in both primordial and primary follicles granulosa cells but appeared in later stages (secondary onward). This study also proved that immunofluorescence labelling combined with intensity quantification using computerized 3D-image analysis was an efficient tool for the *in situ* detection and quantification of the small amount of protein in complex tissues such as ovarian follicles.

As part of the second objective, female Swiss mice, aged 21 days, were passively immunised against BMPR-1B and BMP-4 to examine the role of (BMPs) in the regulation of follicle development including the primordial follicles recruitment, primary follicles survival, ovulation rate and the interaction with gonadotropin hormones. While a stereological study revealed that the number of primordial follicles in immunised mice was significantly higher compared to control animals, treatment with eCG showed no effect. In parallel, immunofluorescence microscopy examination revealed that the presence of BMPR-1B and the lack of FSH receptors in follicle cells of the primordial follicles. Furthermore, estimated numbers of primordial and primary follicles in immunised mice were significantly increased when compared with control animals. After puberty, the rates of depletion of the primordial and primary follicles were increased with age, particularly in treated animals; however, there was no significant difference between the treatment groups of the same age. The results of this study showed that the passive immunisation against BMP signalling can be used as a practical approach in sustaining the reproductive reserve in mammals. In addition, this study demonstrated, for the first time, that the BMP signalling is involved directly in regulating the ovulation process by increasing the number of corpus lutea.

As part of the third objective, the detailed mechanism of interaction between the gonadotropin and the BMP signalling system were explored. Female mice were passively immunised against BMPR-1B and BMP-4 and also administrated with eCG. This study proved that BMPs are not only involved directly in regulating the early stages of follicle development but also involved indirectly in regulating the later stages of follicle development by increasing gonadotropin receptor expression, which ultimately increase the sensitivity of the follicular somatic cell (granulosa and theca cells) toward gonadotropin hormones.

As a part of the fourth and last objective of the study, the interaction between the BMP signalling and gonadotropin hormone receptors expression was investigated at the transcript level through quantitative real-time (qPCR). This study was also conducted in mice passively immunised against BMP receptor 1B (BMPR-1B) and BMP-4 and also administrated eCG. The outcomes of this study showed that attenuation of the BMP signalling significantly increase the mRNA expression of the gonadotropin receptor in mice treated with anti-BMPR-1B and mice administrated with eCG respectively. Meanwhile, mice treated with anti-BMP-4 showed no effects on the gonadotropin receptors expression. This observation, which requires further investigation, suggested that the interaction between the BMP signalling and gonadotropin hormones occurs at intracellular level (transcript level) and not extracellular level.

Collectively, all these results generated in this thesis suggest a new strategy for enhancement of the reproductive life in mammals through increases in primordial follicle reserve, primary follicle survival rate, decreased atresia and increased ovulation rate. Furthermore, the outcome of this study showed that BMPs are involved in increasing the follicular somatic cell sensitivity toward gonadotropin hormones through increasing the gonadotropin receptors expression for FSH and LH in various stages of ovarian follicle development.

## Acknowledgment

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## List of publications by the candidate Included as part of the thesis

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- **Al-Samerria, S., & Almahbobi, G.** (2014). Three-dimensional Image Analysis to Quantify the Temporo-spatial Expression of Cellular Receptors. *Journal of Medical and Bioengineering*, 3(3), 179-182.
- **Al-Samerria, S., Al-Ali, I., McFarlane, J. R. & Almahbobi, G.** 2015. The impact of passive immunisation against BMPRII and BMP-4 on follicle development and ovulation in mice. *Reproduction*, 149, 403-411.
- **Al-Samerria, S., Al-Ali, I., McFarlane, J. R. & Almahbobi, G.** 2016. Protein expression of BMPR-1B, FSHR and LHR in Granulosa cells of mice after passive immunisation against BMPR-1B and Bmp-4 . *In progress to be submitted*.
- **Al-Samerria, S., Palanisamy S. K. A., McFarlane, J. R. & Almahbobi, G.** 2016. The Impact of impaired function of BMPR-1B on mRNA expression of FSHR and LHR in mice. *in progress to be submitted*.
- **Al-Samerria, S., McFarlane, J. R., Mamotte, C. J. & Almahbobi, G.** 2016. The role of BMP signalling in ovarian follicle development: The story from follicle recruitment to ovulation. *Review. in progress to be submitted*.

## Relevant publications to the thesis but not forming part of it

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- **Weal, B. M., Al-Samerria, S., Conceicao, J., Yovich, J. L., & Almahbobi, G. (2015).** A direct action for growth hormone in improving oocyte quality in poor responder patients. *Reproduction*. doi: 10.1530/rep-14-049.
- **Seet, V. Y., Al-Samerria, S., Wong, J., Stanger, J., Yovich, J. L., & Almahbobi, G. (2013).** Optimising vitrification of human oocytes using multiple cryoprotectants and morphological and functional assessment. *Reprod Fertil Dev*, 25(6), 918-926. doi: 10.1071/rd12136.

## List of conference proceeding and published abstract

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- Conference proceeding

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- ***Al-Samerria, S., McFarlane, J., & Almahbobi, G.*** (2013). Passive immunisation is a useful model to study the role of BMPRs in the regulation of ovarian follicle development in mice. Paper presented at the 56-annual meeting of endocrine society of Australia & Society of reproductive biology ESA-SRB, Sydney /Australia. **(Poster)**
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- ***Al-Samerria, S., McFarlane, J., & Almahbobi, G.*** (2011). The Temporo-Spatial Expression of BMP and FSH Receptors during Early Follicle development in Sheep Ovary. Paper presented at the Mark Liveris Health Science Research Student Seminar, Perth/Australia. **(Oral)**

- Published abstract

- ***Al-Samerria, S., McFarlane, J., & Almahbobi, G.*** (2014). The impact of Passive immunisation against bone morphogenetic protein signalling BMPs on female fertility reserve. Paper presented at the European Society of Human Reproduction and Embryology (ESHRE), London/UK. Human Reproduction, 28, i248-i255.



## Statement of contribution of others

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**Sarmed Al-Samerria**  
**1<sup>st</sup> of September 2016**

## Abbreviation

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<b>AA</b>	<b>Amino acids</b>
<b>ActR</b>	<b>Activin receptor</b>
<b>ALK</b>	<b>Activin receptor like kinases</b>
<b>AMH</b>	<b>Anti-mullerian hormone</b>
<b>ANOVA</b>	<b>Analysis of variance</b>
<b>ART</b>	<b>Reproductive-related technology</b>
<b>BAMBI</b>	<b>BMP and activin membrane bound inhibitor</b>
<b>BMPs</b>	<b>Bone morphogenetic proteins</b>
<b>BMPR</b>	<b>BMP receptor</b>
<b>cAMP</b>	<b>Cyclic adenosine monophosphate</b>
<b>cDNA</b>	<b>Complimentary DAN</b>
<b>COC</b>	<b>Cumulus oocyte complex</b>
<b>Co-Smad</b>	<b>Common Smad</b>
<b>CL</b>	<b>Corpus luteum</b>
<b>DNA</b>	<b>Deoxyribonucleic acid</b>
<b>E2</b>	<b>Estradiol</b>
<b>eCG</b>	<b>Equine chorionic gonadotrophin</b>
<b>EGF</b>	<b>Epidermal growth factor</b>
<b>ELISA</b>	<b>Enzyme linked immunosorbent assay</b>
<b>FCA</b>	<b>Freud's complete adjuvant</b>
<b>bFGF</b>	<b>Basic Fibroblast growth factor</b>
<b>FFPE</b>	<b>Formalin fixed paraffin embedded</b>
<b>FSH</b>	<b>Follicle stimulating hormone</b>

<b>FSHR</b>	<b>Follicle stimulating hormone receptor</b>
<b>FSH-RF</b>	<b>FSH-releasing factor</b>
<b>GDF</b>	<b>Growth differentiation factor</b>
<b>GDP</b>	<b>Guanosine diphosphate</b>
<b>GS</b>	<b>Goat serum</b>
<b>GTP</b>	<b>Guanosine triphosphate</b>
<b>GnRH</b>	<b>Gonadotrophin-releasing hormone</b>
<b>HOTT</b>	<b>Human ovarian theca tumor cells</b>
<b>IF</b>	<b>Immunofluorescent</b>
<b>Ig</b>	<b>Immunoglobulin</b>
<b>IGF</b>	<b>Insulin-like growth factor</b>
<b>IU</b>	<b>International units</b>
<b>I-Smad</b>	<b>Inhibitor Smad</b>
<b>KL</b>	<b>Kit ligand</b>
<b>LH</b>	<b>Luteinising hormone</b>
<b>LHR</b>	<b>Luteinising hormone receptor</b>
<b>Liquid nitrogen</b>	<b>LN<sub>2</sub></b>
<b>MAPK</b>	<b>Mitogen activated protein kinase</b>
<b>MIS</b>	<b>Mullerian inhibiting substance</b>
<b>mRNA</b>	<b>Messenger RNA</b>
<b>PBS</b>	<b>Phosphate buffered saline</b>
<b>PCR</b>	<b>Polymerase chain reaction</b>
<b>PFD</b>	<b>Paraformaldehyde</b>

<b>PGC</b>	<b>Primordial germ cell</b>
<b>PKA</b>	<b>protein kinase A</b>
<b>qPCR</b>	<b>Quantitative polymerase chain reaction</b>
<b>RNA</b>	<b>Ribonucleic acid</b>
<b>RS</b>	<b>Rabbit serum</b>
<b>R-Smad</b>	<b>Receptor regulated Smads</b>
<b>Smurf</b>	<b>Smad ubiquitination regulatory proteins</b>
<b>STAR</b>	<b>Steroidogenic acute regulatory protein</b>
<b>TF</b>	<b>Transcription factors</b>
<b>TGF</b>	<b>Transforming growth factor</b>
<b>TGF-<math>\beta</math></b>	<b>Transforming growth factor beta</b>
<b>TNF-<math>\alpha</math></b>	<b>Tumor necrosis factors alpha</b>

## List of contain

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### Contents

Declaration .....	2
Abstract .....	3
Acknowledgment .....	6
List of publications by the candidate Included as part of the thesis .....	7
Relevant publications to the thesis but not forming part of it .....	7
List of conference proceeding and published abstract .....	8
• Conference proceeding .....	8
• Published abstract.....	8
Statement of contribution of others .....	9
Abbreviation.....	10
List of contain.....	13
List of Figures .....	18
List of tables.....	20
Chapter 1 : The female reproductive system .....	21
1.1 Overview .....	21
1.2 Anatomy and Histology of the Ovary .....	21
1.2.1 Primordial follicles.....	22
1.2.2 Primary follicles.....	23
1.2.3 Secondary Follicles .....	24

1.2.4 Graafian follicles .....	25
1.2.5 The Oocyte .....	26
1.2.6 Granulosa cell.....	27
1.2.7 Theca cell.....	28
1.3 Follicle development and function (Folliculogenesis) .....	29
1.4 Gonadotropin hormones and Hypothalamus pituitary-gonadal axis.....	30
1.4.1 FSH Action in the Granulosa Cells .....	31
1.4.2 LH Action in Theca Interstitial Cells.....	33
1.5 Ovulation and atresia.....	34
Chapter 2 : The Transforming Growth Factor (TGF- $\beta$ ) Superfamily .....	35
2.1 TGF- $\beta$ Receptors.....	35
2.1.1 Structure of TGF- $\beta$ Receptors .....	35
2.2 The BMPs .....	38
2.4.1 Members of BMP family .....	39
2.4.1 BMP-1.....	40
2.4.2 BMP-2 (BMP-2A or BMP-2 $\alpha$ ).....	40
2.4.3 BMP-4 (BMP-2B or BMP-2 $\theta$ ).....	40
2.4.4 BMP-5.....	41
2.4.5 BMP-6 (VGR-1).....	41
2.4.6 BMP-7 (OP-1) .....	42
2.4.7 BMP-15 (GDF-9B).....	42
2.4.8 GDF-5.....	43
2.4.9 GDF-9.....	44
2.3 BMP Signal Transduction .....	45

2.4 Factors affecting BMP Signalling .....	47
2.4.1 Intracellular regulatory Factors .....	47
2.4.2 Extracellular regulatory Factors.....	48
2.5 The Regulatory role of BMPs in the Ovary.....	49
2.6 Sheep breeds with a high ovulation rate .....	53
2.7 The impact of mutation of functional (BMPs) on female reproduction.....	54
2.8 The interplay between gonadotropin and BMP in folliculogenesis .....	56
Chapter 3 : Three-dimensional image analysis to quantify the Temporo-spatial expression of cellular receptor .....	
4.3 Materials and Methods .....	59
4.4 Results.....	60
4.5 Discussion.....	61
4.6 References.....	62
Chapter 4 : The Impact of passive immunisation against BMPR-1B and BMP-4 on follicle development and ovulation in mice .....	
3.2 Introduction .....	63
3.1 Abstract.....	63
3.3 Materials and methods .....	64
3.4 Results.....	66
3.5 Discussion.....	67
3.6 References.....	70

Chapter 5 : Protein expression of BMPR-1B, FSHR and LHR in granulosa cells of mice after passive immunisation against BMPR-1B and Bmp-4 .....	72
5.1 Abstract .....	73
5.2 Introduction .....	74
5.3 Materials and methods .....	76
5.3.1 <i>Animal care and passive immunisation</i> .....	76
5.3.2 <i>Antibodies</i> .....	76
5.3.3 <i>Immunofluorescent labelling of BMPR-1B, FSH and LH receptors</i> .....	77
5.3.4 <i>Image analysis and Quantification of Immunofluorescent Intensities of the receptors</i> .....	77
5.3.5 <i>Statistical analysis</i> .....	78
5.4 Results.....	78
5.4.1 <i>Immunofluorescent localisation of receptors in ovarian follicle</i> .....	78
5.4.2 <i>In situ quantification of BMPR-1B, FSHR, and LHR in three different stages of follicle development</i> .....	79
5.5 Discussion.....	80
5.6 Acknowledgement .....	83
5.7 References.....	84
5.8 Figures & Figure Legends .....	87
Chapter 6 : The Impact of impaired function of BMPR-1B on mRNA expression of FSHR and LHR in mice. ....	92
6.1 Abstract .....	93
6.2 Introduction .....	94



6.3 Materials and methods .....	96
6.3.1 <i>Passive immunisation and experimental animals' growth condition</i> .....	96
6.3.2 <i>Antibody preparation</i> .....	97
6.3.3 <i>RNA extraction and RT-PCR</i> .....	97
6.3.4 <i>Primers and qPCR</i> .....	98
6.3.5 <i>Statistical analysis</i> .....	98
6.4 Results.....	99
6.4.1 <i>Relative expression of transcripts of FSHR and LHR after different treatments</i> .....	99
6.5 Discussion.....	99
6.6 Acknowledgements.....	101
6.7 References.....	101
6.8 Figure and figure legends.....	103
Chapter 7 : General discussion .....	104
Chapter 8 : Conclusion .....	108
Chapter 9 : References.....	109
Appendix .....	124

# List of Figures

FIGURE 1-1 REPRESENTATION OF THE STRUCTURE OF THE ADULT OVARY IN HUMAN .....	22
FIGURE 1-2 DIAGRAMMATIC REPRESENTATION OF A PRIMORDIAL FOLLICLE'S STRUCTURE.....	23
FIGURE 1-3: <i>DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF A PRIMARY FOLLICLE</i> .....	24
FIGURE 1-4 DIAGRAMMATIC REPRESENTATION OF SECONDARY FOLLICLE.....	25
FIGURE 1-5: DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF A GRAAFIAN FOLLICLE.....	26
FIGURE 1-6: STRUCTURAL DIAGRAM REPRESENTS HUMAN'S OOCYTE .....	27
FIGURE 1-7: ESTRADIOL PRODUCTION IN GRANULOSA CELLS.....	28
FIGURE 1-8: <i>HISTOLOGICAL PRESENTATION OF THECA CELLS IN A GRAAFIAN FOLLICLE</i> .....	29
FIGURE 1-9: SCHEMATIC REPRESENTATION OF FOLLICULOGENESIS PROCESS IN HUMANS.....	30
FIGURE 1-10: THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS .....	31
FIGURE 1-11: <i>ILLUSTRATION OF THE FSH SIGNAL TRANSDUCTION PATHWAY IN THE GRANULOSA CELLS</i> .....	32
FIGURE 1-12: <i>REGULATORY MECHANISMS OF ESTROGEN PRODUCTION BY THECA INTERSTITIAL CELLS</i> .....	33
FIGURE 2-1: <i>SMAD-DEPENDENT TGF-<math>\beta</math> SIGNALLING PATHWAY</i> .....	36
FIGURE 2-2: <i>BMP SIGNAL TRANSDUCTION PATHWAY</i> .....	46
FIGURE 2-3: <i>NUMBER OF PRIMORDIAL FOLLICLES IN BOORoola/MERINO SHEEP AT BIRTH, 1.5 YEARS AND 5 YEARS</i> .....	54
FIGURE 2-4: <i>MUTUAL REGULATION OF THE BMP SYSTEM AND FSH SIGNALLING IN GRANULOSA CELLS</i> .....	57
FIGURE 5-1: THE EXPRESSION OF BMPR-1B, FSHR AND LHR USING IMMUNOFLUORESCENCE LOCALISATION IN GRAAFIAN FOLLICLES. .....	87
FIGURE 5-2: IMMUNOFLUORESCENCE QUANTIFICATION.....	88
FIGURE 5-3: <i>IN SITU</i> QUANTIFICATION OF THE EXPRESSION OF RECEPTORS (PIXEL/MM <sup>2</sup> ) IN GRAAFIAN FOLLICLES AFTER DIFFERENT TREATMENTS. NUMBER OF FOLLICLES N=20.....	89
FIGURE 5-4: <i>IN SITU</i> QUANTIFICATION OF THE EXPRESSION OF RECEPTORS (PIXEL/MM <sup>2</sup> ) IN SECONDARY FOLLICLES AFTER DIFFERENT TREATMENTS. NUMBER OF FOLLICLES N=20 PER OVARY.....	90
FIGURE 5-5: <i>IN SITU</i> QUANTIFICATION OF THE EXPRESSION OF RECEPTORS (PIXEL/MM <sup>2</sup> ) IN PRIMARY FOLLICLES AFTER DIFFERENT TREATMENTS. NUMBER OF FOLLICLES N=20 PER OVARY.....	91
FIGURE 6-1: RELATIVE GENE EXPRESSION (MRNA) LEVELS OF FSH AND LH RECEPTORS IN WHOLE OVARIES AFTER DIFFERENT TREATMENTS. THE DIFFERENCE WAS CONSIDERED SIGNIFICANT AT.....	103



## List of tables

---

TABLE 2-1: <i>THE RELATIONSHIPS BETWEEN LIGANDS, RECEPTORS AND SMADS OF THE TGF-B SUPERFAMILY</i> .....	37
TABLE 2-2: FUNCTIONAL ANALYSIS OF BMP GENES .....	39
TABLE 2-3: <i>KNOCKOUT PHENOTYPES OF PHENOTYPES OF SEVERAL MEMBERS OF THE BMP SIGNALLING PATHWAY</i> .....	45
TABLE 2-4: THE SECRETORY ACTIVITY OF DIFFERENT FOLLICLE CELL TYPES (M-RNA) OF THECA CELLS, GRANULOSA CELLS AND OOCYTE- AND ITS RELATION TO INTRA-OVARIAN SIGNALLING MOLECULES, INCLUDING THE BMP FAMILY MEMBERS .....	51
TABLE 5-1: ANIMAL TREATMENT GROUPS .....	76
TABLE 6-1: ANIMAL GROUPS AND TREATMENT .....	96
TABLE 6-2: PRIMERS USED IN THIS STUDY .....	98

# Chapter 1 : The female reproductive system

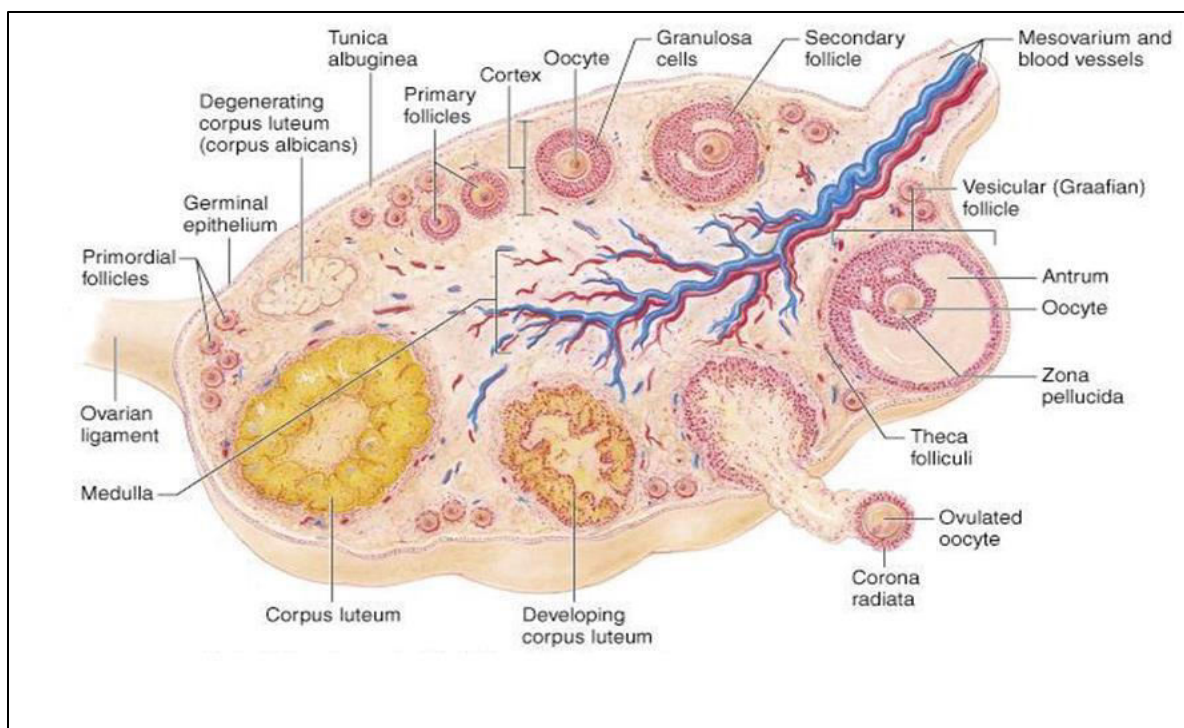
## 1.1 Overview

In mammals, the male (testis) and female (ovary) gonads of newborns contain a finite number of germ cells that serve as a fertility reserve for the whole reproductive age (Ruoss et al., 2009, Monniaux et al., 2014). In the ovary of young females, germ cells (primary oocytes) are incorporated within the primordial follicles; the continuous usage of these follicles results in the depletion of the fertility reserve with age, hence causing ovarian function cessation and infertility (McGee and Hsueh, 2000). The two gonadotrophic hormones, (FSH) and (LH), reported to have a primary role in controlling ovarian function including follicle development (Spiteri-Grech et al., 1993, McNeilly et al., 2003, Castilho et al., 2014). The primordial follicles recruitment, which was thought to be mainly regulated by gonadotropin hormones, was recently shown to be gonadotropin-independent in mice using FSH gene knockout mice (Siegel et al., 2013). Therefore, the role of gonadotropic hormones in the regulation of ovarian function is now believed to be more complex and influenced by the interactions between the gonadotropin and many other intra-ovarian factors, such as BMPs (Shimasaki et al., 2004, Wang and Roy, 2009, Shi et al., 2010, Guangqin et al., 2013). BMPs are classified as members of transforming growth factor beta (TGF- $\beta$ ) superfamily, that interact by binding to their serine/threonine-specific kinase receptors (Rahman et al., 2015) to generate intracellular signals by using the intra-cytoplasmic transcription factors SMADs (Pangas, 2012) to regulate the basic biological processes and development of the immune system. The present study will therefore, focus on the role and significance of BMPs in ovarian function by exploring the interaction between BMP signalling and gonadotropin hormones (FSH and LH) using several technical approaches in different animal experimental model.

## 1.2 Anatomy and Histology of the Ovary

The ovary is a pair of intra-abdominal organs whose most important function is to release a viable and fertilizable egg in a regular cycle (Gougeon, 1996) throughout the process of folliculogenesis. Folliculogenesis process ultimately resulting in ovulation, which is a continuous dynamic process, with some aspects that take place even during pregnancy (Peters et al., 1975), albeit ending at an earlier phase of follicle development. The structure of the adult human ovary can be subdivided into three distinct regions (**Figure 1-1**) (Bloom W

and Fawcett D, 1975): the cortex, the medulla and the helium. The cortex comprises the surface epithelium, tunica albuginea, ovarian follicle at numerous stages of development and the corpus luteum (Bloom W and Fawcett D, 1975). The medulla encompasses large blood vessels and nerves (Bloom W and Fawcett D, 1975). The hilum is the place where the large vessels and nerves leave and enter the ovary consisting of large arteries and Leydig cells (Bloom W and Fawcett D, 1975). Different studies in the past decade have demonstrated that the ovarian function and follicle development to be regulated by several members of the TGF- $\beta$  superfamily such as inhibin, BMPs and activin (Findlay et al., 2002, Wordinger and Clark, 2007).



**Figure 1-1 Representation of the structure of the adult ovary in human**

Sourced from (<http://www.pathologyoutlines.com/topic/ovarynontumornormalhistology.html>)

### **1.2.1 Primordial follicles**

In mammals, the primordial follicles considered as the corner stone in the female reproductive system. It comprises in 90-95% of the ovarian follicle (Becker, 2001). Histologically, the human primordial follicles (~15-25  $\mu\text{m}$  in diameter) contain a small primary oocyte surrounded by a single layer of squamous cells and a basal lamina (Saitou et al., 2002) (**Figure 1-2**). Primordial follicles in humans are produced in the fetus during the last trimester of pregnancy and hence all oocytes capable of taking part in reproduction already present at birth (Kezele and Skinner, 2003). Primordial follicles are regulated by the paracrine/endocrine

network of communications between the oocyte, attached granulosa cells, nearby theca/interstitial cells as well as surrounding follicles, in addition to the influence of several intra-ovarian growth factors such as TGF- $\beta$  superfamily (Shimasaki et al., 2004). The first step in folliculogenesis is the primordial/primary follicle transition which includes activation and growth of a dormant primordial follicle (Fortune, 1994). The first sign of activation of the primordial follicle is the changes in its shape from squamous to cuboidal cells morphology. The granulosa cells begin to multiply leading to follicle growth and development (Shimizu et al., 2004).

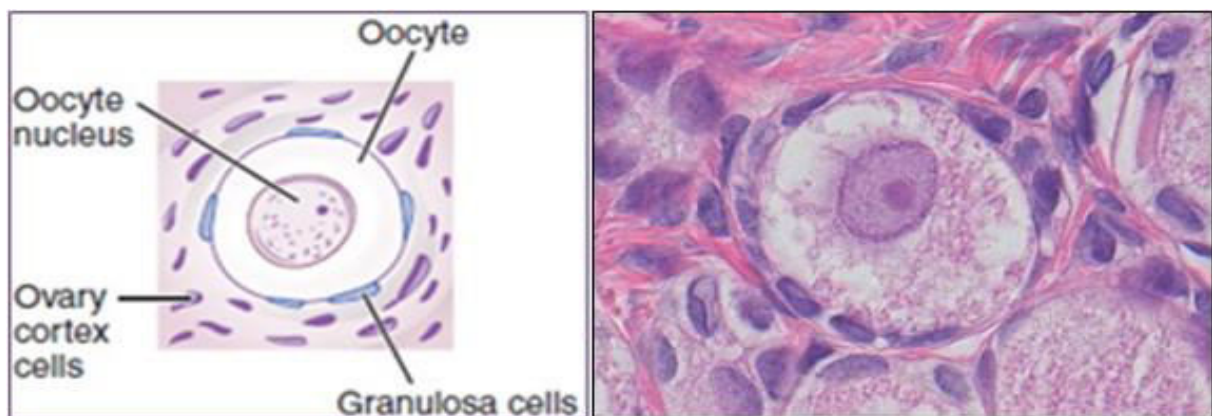


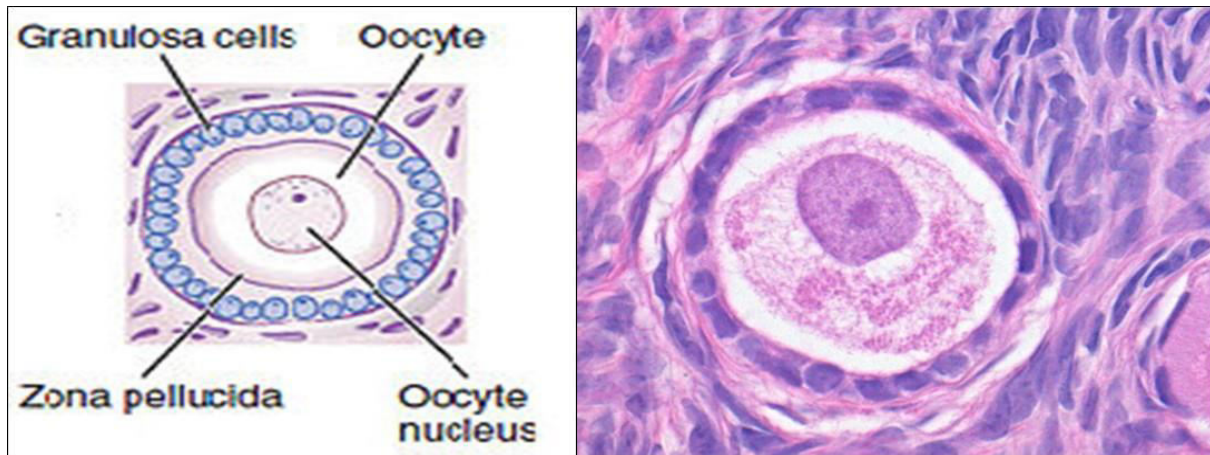
Figure 1-1-2 Diagrammatic representation of a primordial follicle's structure

Sourced from: (<https://www.studyblue.com/notes/note/n/reproductive-system/deck/2542864>)

### 1.2.2 Primary follicles

The primary follicle consists of a cuboidal granulosa cells that are aligned in a single layer to surround and enclose the oocyte (**Figure 1-3**). The expression of FSH receptor in granulosa cells as well as oocyte growth and development considered as the main event that takes place in the primary follicle development (Williams and Erickson, 2008). The formation of a second layer of granulosa cells is exclusive to mammals and termed the primary/secondary follicle transition (Dodd, 1977).

Electron microscope examination of primary follicle oocytes in humans, and compared to primordial follicles, reveals an increase in size, in the number of mitochondria and with a peripheral cytoplasmic location, the development of Golgi apparatus as several groups of smooth vesicles and tubules, and increases in RNA and protein synthesis (James et al., 1976, Gougeon, 1996).



**Figure 1-3: Diagrammatic representation of the structure of a Primary follicle**

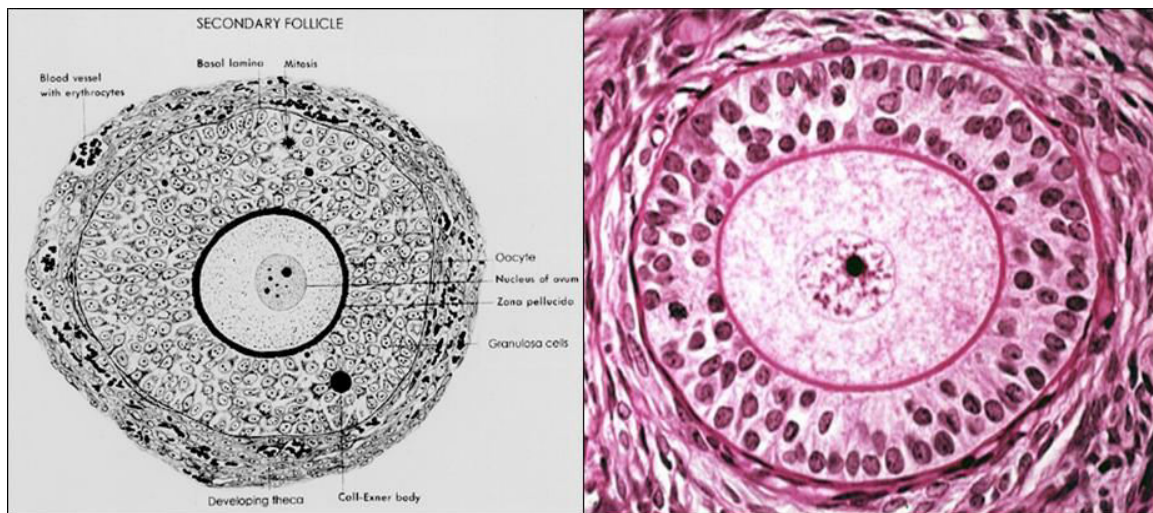
Sourced from (<http://pixgood.com/primordial-follicle.html>)

During the maturation of the oocyte, a small patch of oocyte-derived factors are observed as deposits around the oocyte forming a layer of extracellular matrix called the zona pellucida (ZP) (Becker, 2001). The human ZP is composed of three glycoproteins: ZP-1, ZP-2 and ZP-3 (Moos et al., 1995). ZP-3 acts as main sperm receptors and also induce the acrosome reactions (Wassarman, 1988). The anti-ZP-3 antibody can block fertilization and hence, has implications to be used as a contraceptive vaccine (Levy, 2011, Skinner et al., 1996). Continuous development of the primary follicles leads to increases in its size and number of gap junction (GJ) between the granulosa cells and the oocyte (Matzuk, 2000). The gap junction is comprised of a family of proteins called Connexins (CX) (Becker, 2001). The main function of the (GJ), primarily consisting of CX-34 and CX-37, is the maintenance of the cytoplasmic communications between neighbouring cells (Gershon et al., 2008). Studies in animal ovarian follicle revealed that CX-37 is predominantly present in (GJ) between adjacent granulosa cells, while CX-43 is present in (GJ) between granulosa cells and the oocyte (Kumar and Gilula, 1996). Studies from CX-37 gene knockout mice revealed that CX-37 is essential for follicle development and fertilization (Simon et al., 1997); however, and while their precise role in follicle development is yet to be well determined (Teilmann, 2005).

### **1.2.3 Secondary Follicles**

The development of secondary follicles starts when the primary follicles gain additional layers of granulosa cells as the alignment of the granulosa cells changes from a simple cuboidal to a pseudostratified epithelium cells (Williams and Erickson, 2008) (**Figure 1-4**).





**Figure 1-4 Diagrammatic representation of secondary follicle**

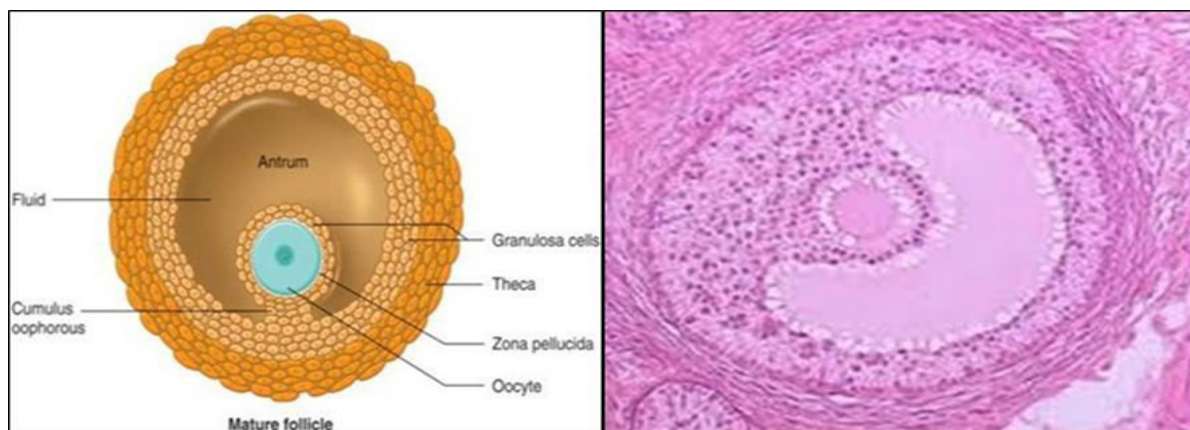
Sourced from: <http://pictures.doccheck.com/com/photo/9949-human-oocyte-secondary-follicle>)

After the primary to secondary follicle transition, 2-8 layers of granulosa cells surround the oocyte. A “yet to be identified” signal that stimulates the mesenchymal cells migration toward the basal lamina and its division into two primary layers of thecal cells: an inner theca interna and an outer theca externa takes place at this stage of development (Erickson et al., 1985, Becker, 2001). The theca externa consists of the contractile proteins such as actin and myosin (involved in cell movement), whereas the theca interna differentiates into glandular/endocrine theca interstitial cells that secrete androgens (Shimasaki et al., 2004). The androgen secreted from the theca interna was illustrated to be an obligatory precursor for follicle estrogen generation as well as follicle atresia (Erickson et al., 1985). In addition, thecal development is associated with the formation of several small blood vessels around the developing follicle. The circulating blood transports nutrients and gonadotrophins to the developing follicle and removes waste products secreted from the follicle (Williams and Erickson, 2008). Another major event during the secondary follicle development is the formation of an eosinophilic fluid-filled space between the granulosa cells called the Call-Exner bodies (Becker, 2001); histological examination reveals that the Call-Exner bodies are made up of extracellular matrix which is involved in cell proliferation and differentiation (Meredith et al., 1992).

### **1.2.4 Graafian follicles**

Similar to the secondary follicle, Graafian follicles also consist of an oocyte, theca cells and granulosa cells and is distinguished by the presence of an antrum cavity containing follicle fluid adjacent to the oocyte (Williams and Erickson, 2008) (**Figure 1-5**). The antrum contains

molecules produced from the oocyte and granulosa cells that possess a regulatory effect on follicle development (Edwards, 1974). There are two major proteins expressed in follicle fluids which were shown to be vital for antrum development: the granulosa-derived kit ligand and the CX-37 (Teilmann, 2005, Thomas and Vanderhyden, 2006). The Graafian follicle can increase in size by as much as ~75 fold. For instance, in humans, the Graafian follicles can increase from ~0.4 mm to ~30 mm in diameter (McNATTY et al., 1979). The increase in size is characterized by granulosa cells proliferation and the accumulation of the follicle fluid (Edwards, 1974). The granulosa cells align in specifically shaped and positioned cells, such an alignment gives rise to a diverse subtype of granulosa cell structures such as the granulosa membrane (mural granulosa), the pre-antral area and the cumulus oophorus (Becker, 2001).

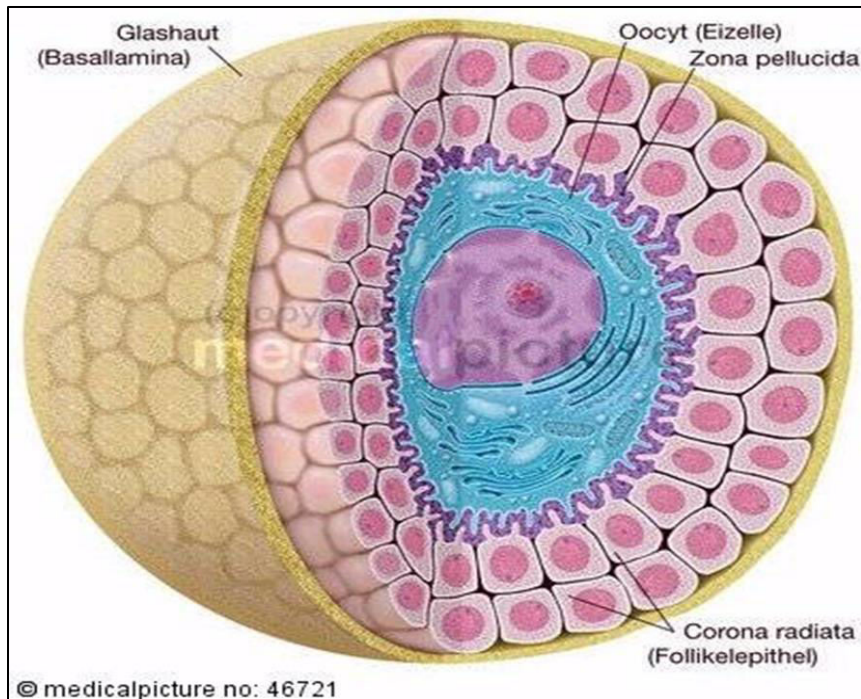


**Figure 1-5: Diagrammatic representation of the structure of a Graafian follicle**

Sourced from: (<http://biology4isc.weebly.com/1-human-reproduction.html>)

### 1.2.5 The Oocyte

The ovaries of adult mammals consist of a large number of primordial follicles containing primary oocytes arrested in the first meiotic prophase stage of division (Wassarman P and Albertini D, 1994) (**Figure 1-6**). After puberty and upon each estrous cycle, mature oocytes resume meiosis and ultimately undergo ovulation (Wassarman P and Albertini D, 1994). The maturation of an oocyte, as an integrated process of folliculogenesis, is regulated by several factors, predominantly the pituitary gonadotrophin hormones (Wassarman P and Albertini D, 1994). The meiotic maturation of an oocyte entails the transformation of immature of an oocyte to a fully grown oocyte presents in the pre-ovulatory follicles ready for fertilization just before ovulation (Wassarman P and Albertini D, 1994). This process is regulated by the interaction of pituitary gonadotrophins with a variety of autocrine and paracrine factors of the TGF- $\beta$  superfamily (Findlay et al., 2002).

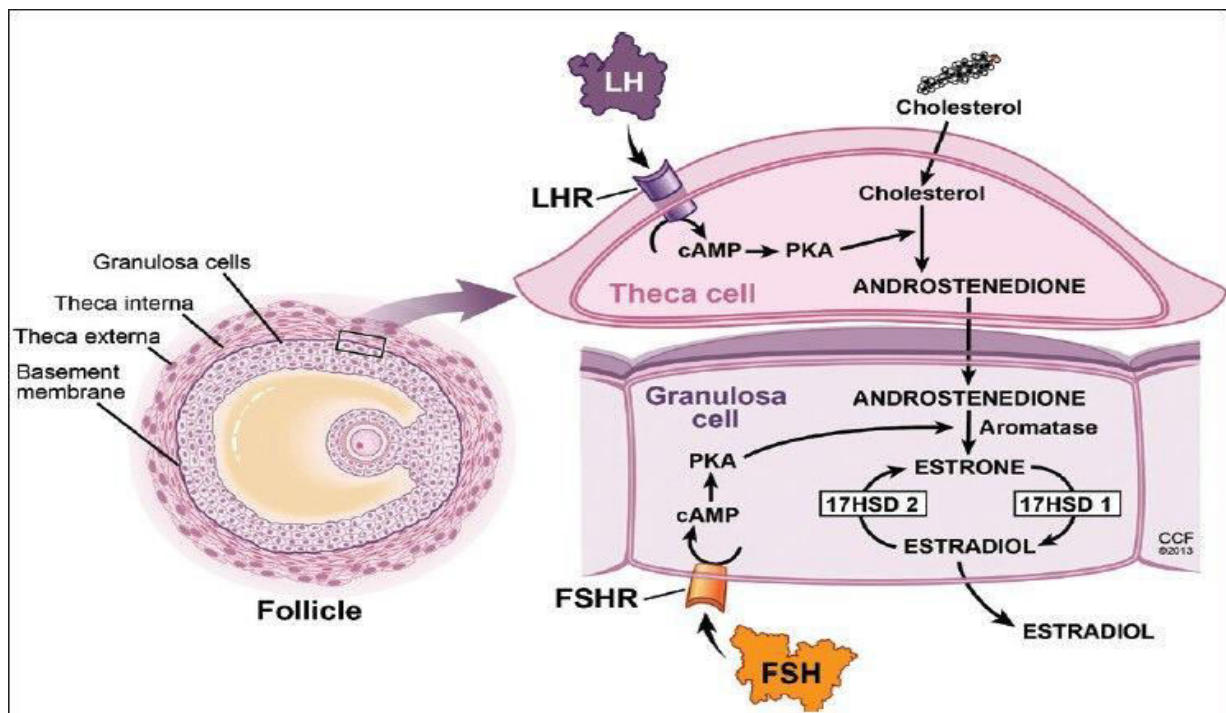


**Figure 1-6: Structural diagram represents human's oocyte**

Sourced from : (<http://pictures.doccheck.com/com/photo/9949/size/m>)

### 1.2.6 Granulosa cell

Granulosa Cells stem from the mesonephric cells throughout embryogenesis, where their number multiplies with follicle growth and affected by levels of estrogen and several intra-ovarian growth factors (Erickson and Danforth, 1995). During folliculogenesis, granulosa cells are unable to produce estrogen until theca cells transform into androgen producing cells. In granulosa cells, the enzyme  $17\beta$ -hydroxysteroid dehydrogenase converts the substrate androstenedione into testosterone, and the enzyme P450-aromatase converts the testosterone produced into estradiol. **Figure 1-7** presents a detailed schematic presentation of estradiol production in granulosa cells.



**Figure 1-7: Estradiol production in granulosa cells**

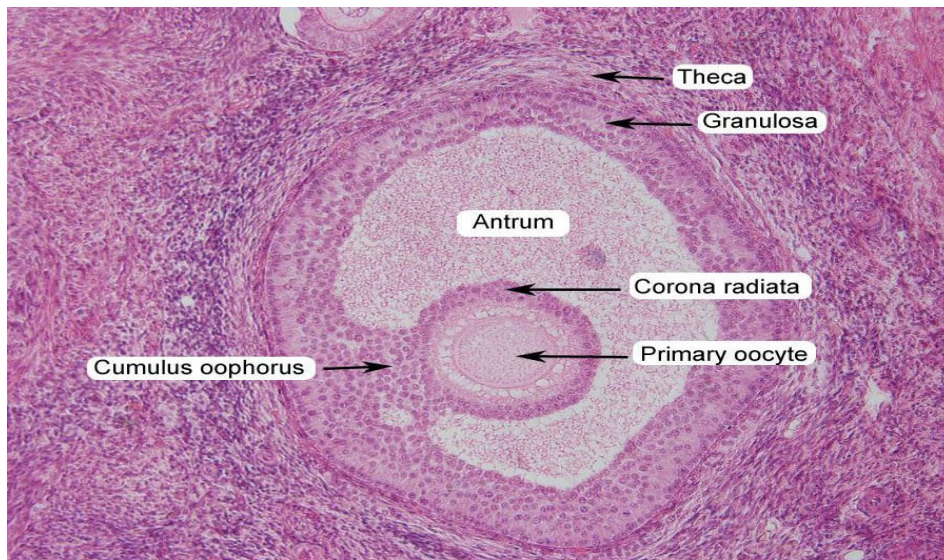
Sourced from: ([https://www.researchgate.net/figure/261140191\\_fig1\\_Two-cell-theory-of-estrogen-production-Luteinizing-hormone-stimulates-the-production-of](https://www.researchgate.net/figure/261140191_fig1_Two-cell-theory-of-estrogen-production-Luteinizing-hormone-stimulates-the-production-of))

Before the mid-cycle gonadotrophin surge that takes place on day-14 of the menstrual cycle, granulosa cells predominantly secrete estradiol. Afterwards, granulosa cells shift to produce progesterone (Erickson and Danforth, 1995). Both FSH and LH augment the production of progesterone from granulosa cells by enhancing the uptake of lipoproteins, freeing cholesterol from lipoproteins, transforming cholesterol into pre-gnenolone and then subsequently converting pre-gnenolone into progesterone (Hsueh et al., 1984).

### 1.2.7 Theca cell

During the secondary stages of follicle development, stromal cells align side by side near the basal lamina to form the theca layer (surrounds granulosa cells) (Gougeon, 1986) ultimately forming an outer and inner sections of theca layer (Gougeon, 1986). The outer section, called the theca externa, comprises undifferentiated theca cells and the inner section, called the theca interna, having similar appearance with the steroid secreting cells (**Figure 1-8**) (Gougeon, 1986). In bovine ovaries, BMP-4 was shown to be situated in the theca interna as well as in the oocyte (Fatehi et al., 2005). The existence of BMP-4 and BMP-7 in thecal cells indicates that these proteins play a role in follicle steroidogenesis since estradiol production requires a close coordination between thecal and granulosa cells (Fatehi et al., 2005).



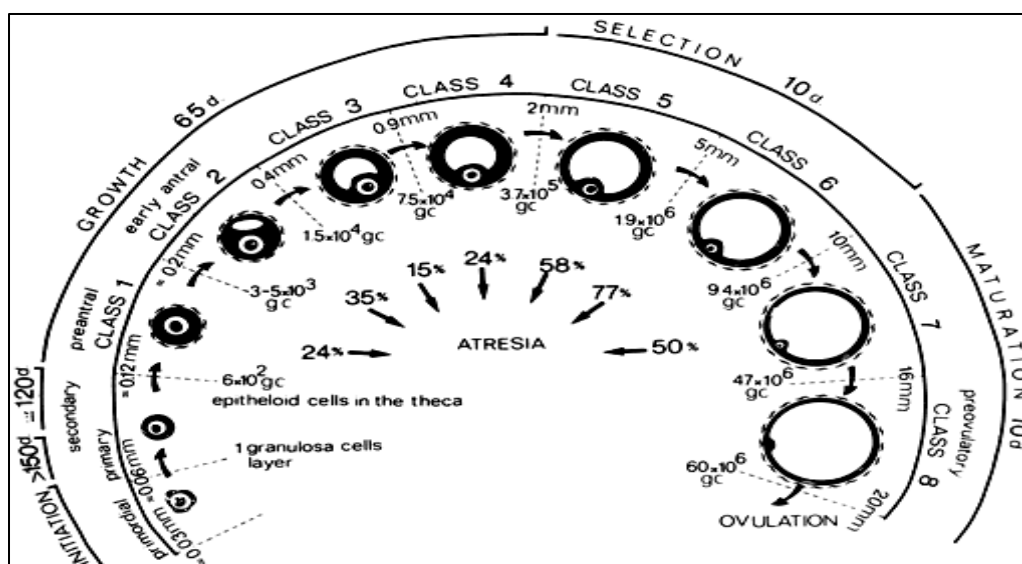


**Figure 1-8: Histological presentation of theca cells in a Graafian follicle**

Sourced from: <http://mesa-anatomy.weebly.com/reproductive1.html>

### 1.3 Follicle development and function (Folliculogenesis)

In humans, folliculogenesis comprises a chronological series of events where growing follicles either mature to ovulation or die by apoptosis (Filicori, 1999, Scaramuzzi et al., 2011, Hsueh et al., 2015). The process of folliculogenesis is a relatively long, and takes approximately one year for a primordial follicle to develop through to the ovulatory stage (Gougeon, 1996, Williams and Erickson, 2008). The early stages comprise the primordial follicle transition, the differentiation and development of follicular somatic cells (granulosa and theca), dominant follicle selection, ovulation, followed by corpus luteum formation and/or atresia (Roche, 1996, Shimasaki, 2006, Shimizu et al., 2004, Hutt and Albertini, 2007, Field et al., 2014). The recruitment of primordial follicle to the pool of growing follicles is considered to be a key limiting step in folliculogenesis process (Fortune et al., 2000). Folliculogenesis takes place in two phases: the first phase is the pre-antral phase which requires around 290 days – includes the characteristic development of the oocyte and the development of a recruited primordial follicle into a primary follicle (Gougeon, 1996, Williams and Erickson, 2008). The second phase is the antral phase that requires around 60 days, where antrum formation is initiated followed by all the developmental changes up to the Graafian follicle stage. Generally, one or two dominant follicles are selected from a large antral group after which it takes around 20 days for the dominant follicle to reach ovulation (**figure 1-9**) (Gougeon, 1986, Fortune et al., 2000).

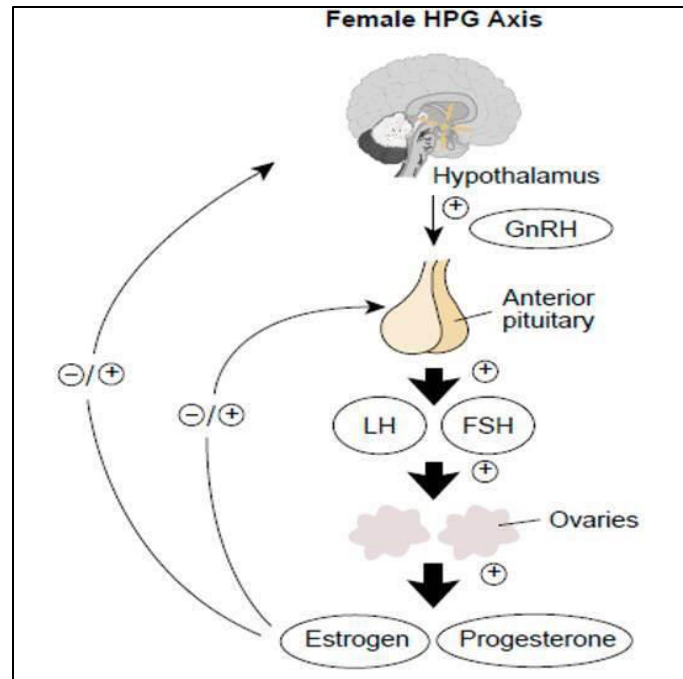


**Figure 1-9: Schematic representation of folliculogenesis process in humans**

Sourced from: (Williams and Erickson, 2008)

## 1.4 Gonadotropin hormones and Hypothalamus pituitary-gonadal axis

The pituitary gland synthesises and releases of the two gonadotropin hormones, FSH and LH which considered as the main players controlling the functions of the gonads (ovary and testis). Gonadotropins production and release is regulated by a tightly coordinated system, which called hypothalamic-gonadal-pituitary (hypothalamic, pituitary and gonadal axis) (**Figure 1-10**) (Faure et al., 2005). The release of the hypothalamic gonadotropin-releasing hormone (GnRH) from the hypothalamus, in pulses manner, appeared to be the primary stimulus for FSH and LH production and release from the pituitary gland (Faure et al., 2005). The frequency of GnRH pulses exhibits selective effect on FSH and LH secretion, where a high frequency of GnRH pulses stimulates LH secretion and a low frequency of GnRH pulses stimulates FSH secretion (Molter-Gerard et al., 1999). Other factors such as gonadal steroids, progesterone and estradiol also regulate FSH and LH release. FSH plays vital roles in several gonadal functions such as follicle development, proliferation of granulosa cells, LH production and steroid production (Fauser and Van Heusden, 1997). In addition to the influence of GnRH, it is well established that FSH secretion is also regulated by members of the TGF- $\beta$  superfamily such as BMP family (Bilezikjian et al., 2006).



**Figure 1-10: The hypothalamic-pituitary-gonadal axis**

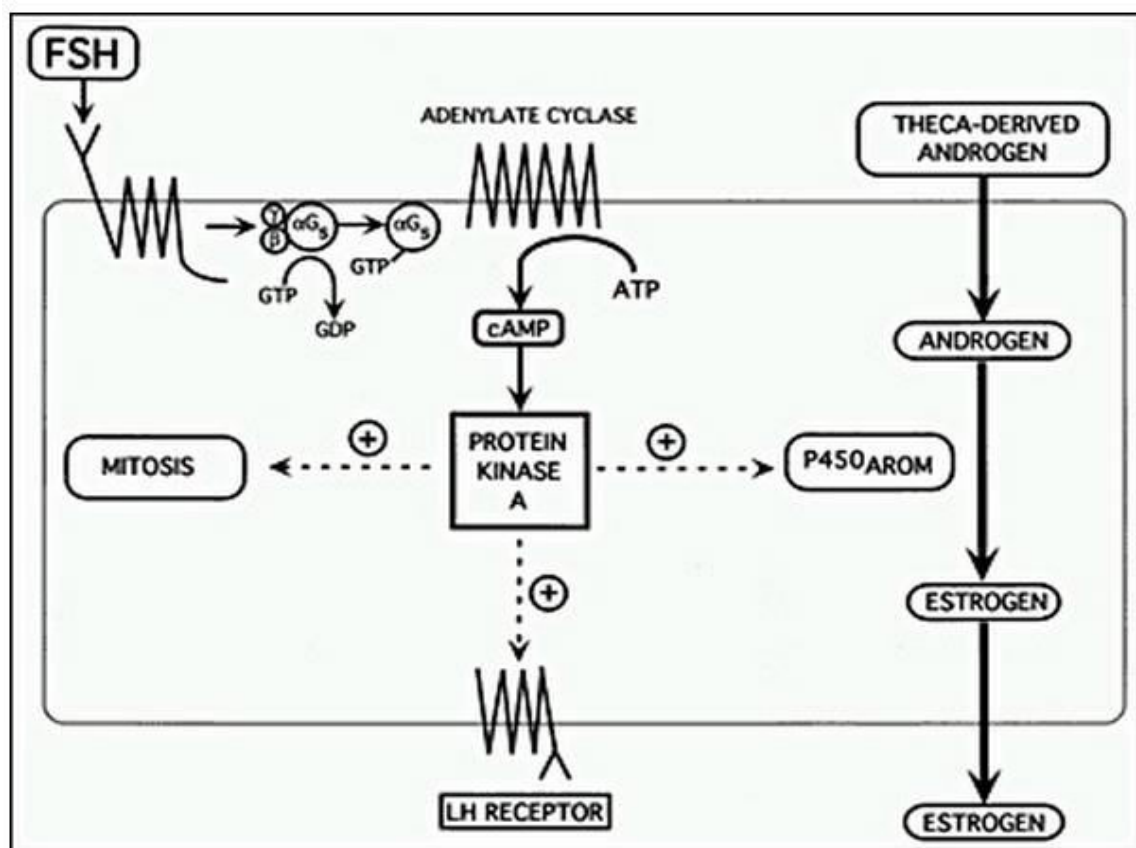
*Sourced from: (Kong et al., 2014)*

#### **1.4.1 FSH Action in the Granulosa Cells**

The human functional FSH-Receptor protein consists of 678 amino acids with three domains: a) a large extracellular amino-terminal ligand binding domain b) the heptahelical transmembrane spanning domain c) and the intracellular carboxyl-terminal domain (Findlay et al., 2002). The primary mechanism by which dominant follicle selection and development take place is through FSH-Receptor pathway activation in the granulosa cells (McGee and Hsueh, 2000, Williams and Erickson, 2008). The FSH signalling pathway is commencing with binding of FSH to its receptor, leading to a conformational change in the transmembrane domain of the receptor (Findlay et al., 2002, Guangqin et al., 2013). Such an alteration leads to the activation of the heterotrimeric G protein (Gs), through an exchange of (GTP) for (GDP). The active (Gs-GTP) subunit activates adenylate cyclase which produces the second messenger cAMP. Consequently, cAMP interacts with protein kinase A (PKA) generating two free catalytic subunits. FSH modulation via the cAMP/PKA pathway in the granulosa cells accounts for dominant follicle growth as well as development to the pre-ovulatory stage (Williams and Erickson, 2008). As illustrated in **(Figures 1-11)**. This is not the only signalling pathway activated by FSH; it has also been shown to activate the cAMP/PKA pathway (Hunzicker-Dunn et al., 2012). Furthermore, FSH was also illustrated to act via the P13K pathway, Src family of tyrosine kinases, G-protein RAS, extracellular related kinases (ERK 1/2) and P38 mitogen-

activated protein kinase (Hunzicker-Dunn and Maizels, 2006). Thus FSH signals through the P13K pathway was suggested to bring the activation of pro-survival factors in granulosa cells (Williams and Erickson, 2008).

FSH is considered the key stimulator of granulosa cell activities in the ovary (Gougeon and Testart, 1990). Additionally, various growth factors produced have a significant influence on granulosa cell proliferation (Williams and Erickson, 2008). For example, oocyte-derived GDF-9 and BMP-15 play a role in granulosa cell activation via modulation of FSH signals (Otsuka et al., 2011). FSH may act synergistically with such growth factors to activate human granulosa cell proliferation (Williams and Erickson, 2008).



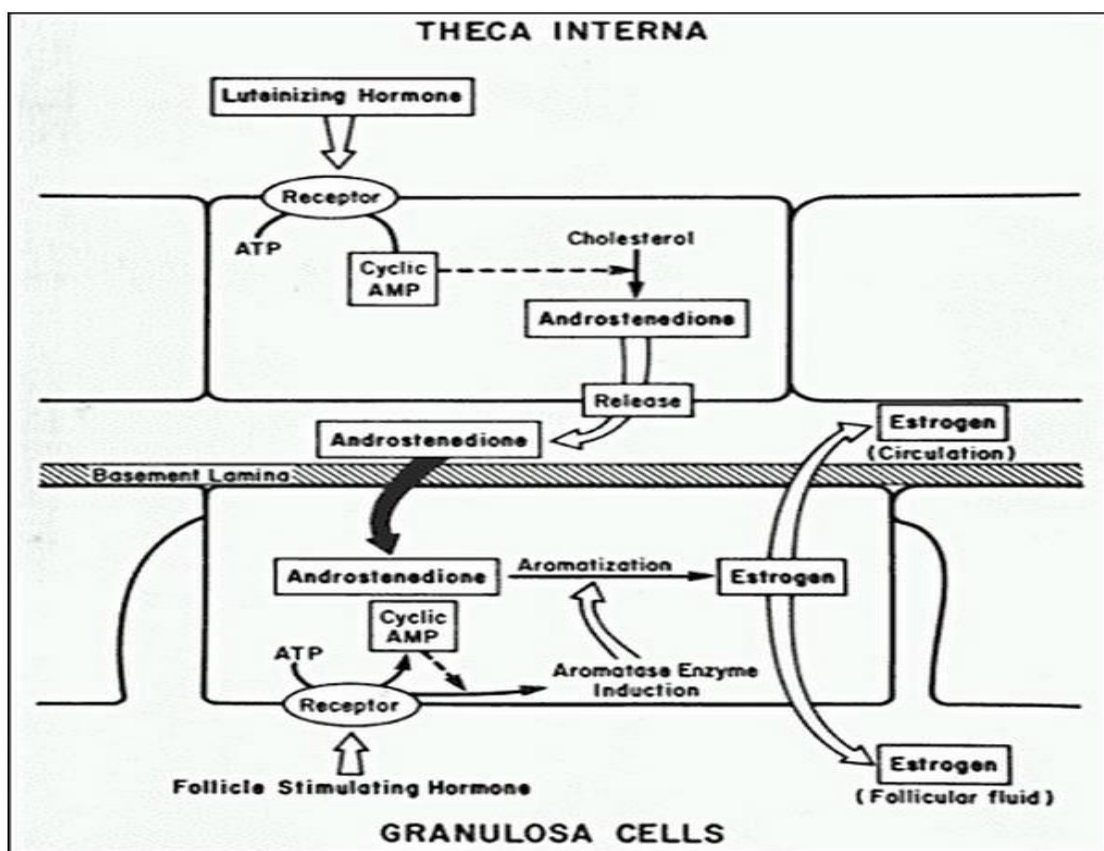
**Figure 1-11: Illustration of the FSH Signal Transduction Pathway in the granulosa cells**

*Sourced from: (Williams and Erickson, 2008)*



### 1.4.2 LH Action in Theca Interstitial Cells

LH-Receptor in human consists of 675 amino acids (Filicori, 1999). Similar to the FSH, LH consists of three domains: a) an extracellular leucine-rich ligand binding domain b) a heptahelical transmembrane domain c) and the intracellular domain for G protein interactions (Filicori, 1999, Williams and Erickson, 2008). However, a number of truncated forms (which determine the biological activities of LH) of the LH receptor not containing the transmembrane domain have also been described (Williams and Erickson, 2008). One of the events that are required for the further development of the pre-ovulatory follicle is the expression of the granulosa cells LH-receptor (McNeilly et al., 2003). The LH receptors remain repressed until late in the follicle cycle and FSH plays an important role in LH-Receptor induction (McGee and Hsueh, 2000, Meldi et al., 2012). Oocyte-derived inhibitors are responsible for blocking the FSH-induced the expression of LH receptors in granulosa cells until the onset of the pre-ovulatory stage (McGee and Hsueh, 2000). Once expressed, LH binds to the LH-Receptor with a high affinity leading to the conformational change in the receptor and activation the G proteins (Filicori, 1999).



**Figure 1-12: Regulatory mechanisms of estrogen production by theca interstitial cells**

Sourced from: (<http://www.sciencedirect.com/science/article/pii/S13572725050005>)

LH receptor activation is similar to FSH receptor activation in that it leads to downstream activation of the Gs/adenylate cyclase/cAMP and PKA pathway (Williams and Erickson, 2008). It was demonstrated that other G proteins (Gi and Gq) may be activated upon LH receptor stimulation to mediate LH-induced activation of phospholipase C and its signalling pathways (Ascoli et al., 2002). Ultimately, stimulation of the LH receptor activates LH signalling pathways that lead to the synthesis of androstenedione (Williams and Erickson, 2008). Figure (1-12) presents an illustration of LH induced activation of the LH receptors and the downstream LH signalling pathways.

### **1.5 Ovulation and atresia**

The human ovaries normally produce a single dominant follicle from growing and developing follicles in each menstrual cycle (McGee and Hsueh, 2000), and this follicle is responsible for enhanced estradiol secretion that occur during the late stages of follicle development, and maturation of the dominant follicle (Williams and Erickson, 2008). After ovulation, the remaining of the dominant follicle develops into the corpus luteum (under influence of LH) and produces large quantities of progesterone throughout the luteal phase of the menstrual cycle (Williams and Erickson, 2008). The estradiol and progesterone generated act synergistically to prepare the uterus for the embryonic implantation (Bazer, 2013). However, continuous follicular atresia and ovulation lead to the complete depletion of ovarian follicles reserve, which ultimately results in ovarian failure and the cessation of menstrual cycle in women. Thus the menopausal state (Ruoss et al., 2009).

Very few of mammalian follicles develop to maturity, with 99.9% of follicles dying due to atresia, the onset of the latter occurring through activation of apoptotic activity in the oocyte and granulosa cells (Ksiazkiewicz, 2006). Apoptosis may be initiated by extracellularly derived ligand/factors (apoptotic factors such as tumor necrosis factor TNF $\alpha$  from epithelia/stromal cells) binding to the granulosa cells or the oocyte surface. Also, apoptosis can be initiated intracellularly by changing the mitochondria's outer membrane permeability to secrete the pro-apoptotic factors into the cytoplasm (Williams and Erickson, 2008, Matsuda et al., 2012). FSH is considered to be a survival factor for Graafian follicles due to its sustained role in follicle growth and inhibitory action on follicles atresia (Craig et al., 2007).

## Chapter 2 : The Transforming Growth Factor (TGF- $\beta$ ) Superfamily

The TGF- $\beta$  superfamily, up to date, comprises of 42 members and can be further subdivided into subfamilies, such as the nodal family, myostatin TGF- $\beta$  family, the inhibin family, the activin family, Mullerian Inhibiting Substance (MIS) and BMPs (Campbell et al., 2012, Rossi et al., 2015). TGF- $\beta$  superfamily have a unique structural feature where the existence of nine conserved cysteine residues, eight of which are involved in forming the characteristic cysteine knot structure of the molecule in the three-dimensional form (Vitt et al., 2001). The ninth conserved cysteine residue is engaged in formation of a di-sulphide bridge between the two subunits, which is responsible for the formation of a covalently linked and biologically active dimer (Jones et al., 1994). All members of TGF- $\beta$  superfamily including that in human, rat, sea urchin, mouse, worm, cattle, zebrafish and chicken have conserved residues, such as valine or leucine or isoleucine at the 31<sup>st</sup> amino acid. Hence, its substitution with an acidic amino acid causes a critical change in its function (Shimasaki et al., 2004).

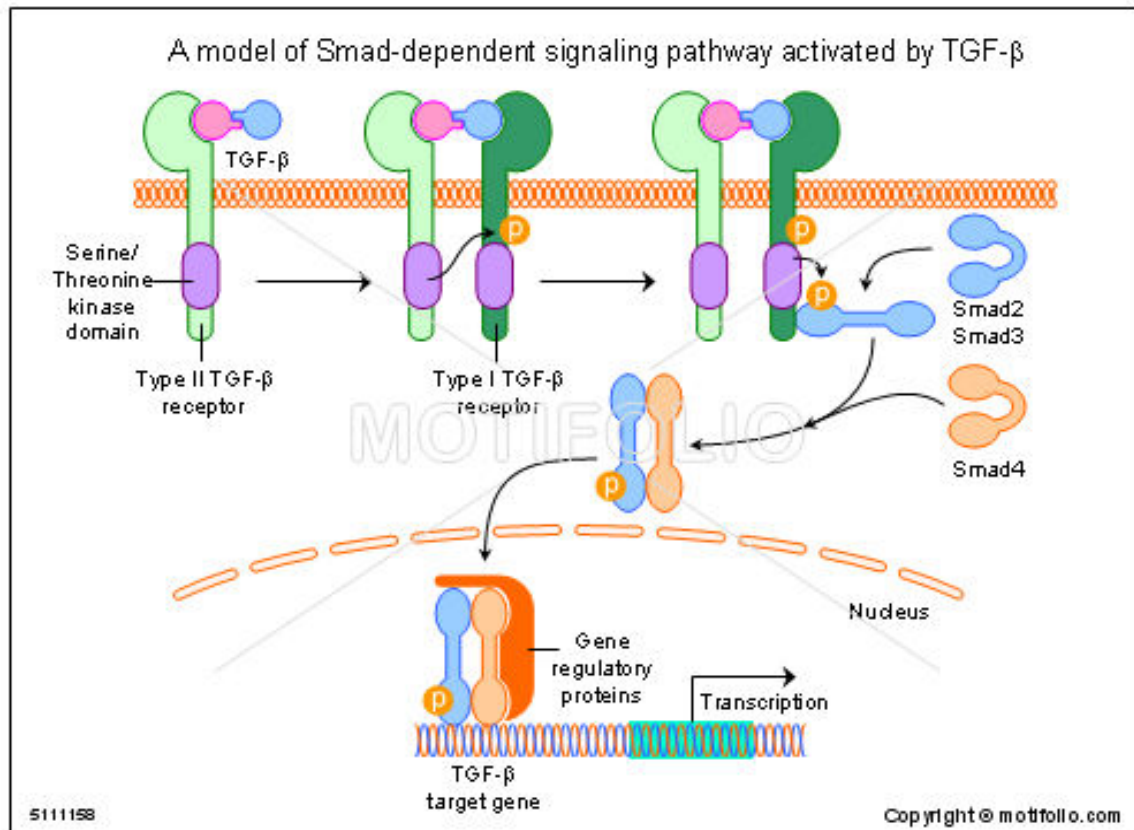
### 2.1 TGF- $\beta$ Receptors

The TGF- $\beta$  superfamily signals through a trans-membrane cellular receptor, which binds to specific ligands, such as hormone growth factors and neurotransmitters (Attisano and Wrana, 2013). The ligand binding causes activation of various intracellular pathways that promotes a particular gene expression which ultimately regulates a specific biological response (Chedrese, 2009). Almost all members of the TGF- $\beta$  superfamily belong to serine/threonine kinase class of receptors (Massague, 1998). The serine/threonine kinase receptors are of two types: type I and type II receptors (Derynck and Zhang, 2003). type I exists in seven forms such as Activin Receptor-Like Kinase, ALKs 1-7, whereas type II exists in five forms, such as T $\beta$ R-II, ActR-II, ActR-IIB, BMPR-II and AMHR-II (Derynck and Zhang, 2003).

#### 2.1.1 Structure of TGF- $\beta$ Receptors

The structure of the TGF- $\beta$  receptor contains three functional domains: (a) extracellular domain, (b) GS domain (transmembrane bounds), and (c) Kinase domain (intra-cytoplasmic) (Massague, 1998). The extracellular domain consists of about 150 amino acids and is concerned with modulating TGF- $\beta$  responses (Lin and Wang, 1992). The GS domain is highly conserved among the type I receptors and consists of 30 amino acids that precede the kinase domain (Lin and Wang, 1992). The GS domain is essential for type I receptor signalling due to

the presence of the phosphorylation site, which is required for the signal transduction. The phosphorylation site in the GS domain is phosphorylated by the type II receptor, which when mutated may prevent the phosphorylation of type I receptor (Attisano and Wrana, 2002). The kinase domain plays a key role in the phosphorylation of Smad proteins (discussed further below) in both type I and type II receptors, thus indicating the importance of the kinase domain in downstream receptor signalling (**Figure 2-1**) (Massague, 1998).



**Figure 2-1: Smad-dependent TGF-β signalling pathway**

Sourced from: (<http://www.motifolio.com/>)

Members of the TGF-β type I receptor family, have high similarity with their kinase domains and signalling properties and are further divided into three groups: (a) ALK-5, ALK-4 and ALK-7; (b) ALK-3 and ALK-6 and (c) ALK-1 and ALK-2 (Chang et al., 2002). TGF-β type II receptor family comprises of three members: (a) TβR-II, (b) BMPR-II and (c) AMHR-II (Chang et al., 2002). **Table 2-1** summarizes the members of TGF-β serine/threonine kinase receptor family. The ligand-receptor binding relationships between several BMP ligands and their respective receptors are not restricted (Shimasaki et al., 2004). ActR-II and ActR-IIB initially discovered as activin receptors were it also act found to act as receptor of BMP-6 (Ebisawa et al., 1999), BMP-7 (Yamashita et al., 1995) and GDF-5 (Nishitoh et al., 1996). By contrast, BMPR-II

receptors bind specifically to BMP ligands, including BMP-2 (Liu et al., 1995), BMP-4 (Nohno et al., 1995), BMP-6 (Ebisawa et al., 1999), BMP-7 (Rosenzweig et al., 1995), BMP-15 (Moore et al., 2003) and GDF-9 (Vitt et al., 2002).

**Table 2-1: The relationships between ligands, receptors and Smads of the TGF- $\beta$  superfamily**  
*Sourced from: (Shimasaki et al., 2004)*

<b>Ligands</b>	<b>Type II-R</b>	<b>Type I-R</b>	<b>Smads</b>
<b>BMP-2</b> <b>BMP-4</b>	BMPR-II	ALK-3 (BMPR-IA) ALK-6 (BMPR-IB)	Smad 1/5/8
<b>GDF-5</b>	BMPRR-II ActR-II ActR-IIB	ALK-3 (ActR-IA) ALK-6(BMPR-IB)	Smad 1/5/8
<b>BMP-6</b> <b>BMP-7</b>	BMPRR-II ActR-II ActR-IIB	ALK-2 (ActR-IA) ALK-6(BMPR-IB)	Smad 1/5/8
<b>BMP-15</b>	BMPRR-II	ALK-6(BMPR-IB)	Smad 1/5/8
<b>GDF-9</b>	BMPRR-II	?	Smad 2
<b>MIS/AMH</b>	AMHR-II	ALK-2 (ActR-IA) ALK-3 (ActR-IA) ALK-6(BMPR-IB)	Smad 1/5/8
<b>Activin</b>	ActR-II ActR-IIB	ALK-4 (ActR-IB)	Smad 2/3
<b>TGF-<math>\beta</math></b>	T $\beta$ R-II	ALK-1 ALK-5(T $\beta$ R-I)	Smad 2/3

In terms of TGF- $\beta$  superfamily type I receptors, ALK-2 (ActR-IA) (Macias-Silva et al., 1998), ALK-3 (BMPR-IA) (Gilboa et al., 2000) and ALK-6 (BMPR-IB) (Aoki et al., 2001) have been illustrated to be similar to BMP type I receptors, where overexpression of these BMP type I receptors activates BMP-specific signalling pathways (Fujii et al., 1999). BMPR-ligand interactions are complex, in that there is much cross-reactivity among various BMP ligands with BMP type I receptors (Shimasaki et al., 2004). To add to this complexity is the specific binding of BMP ligand to different BMP type I receptors in different types of cells (Shimasaki et al., 2004). For instance, in C2C12 and MC3T3-E1 cells, BMP-6 binds to ActR-IA and BMPR-IA, but with higher affinity to ActR-IA (Ebisawa et al., 1999). In ROB-C26 cells, BMP-6 binds to ActR-IA, BMPR-IA

and BMPR-IB, but with a higher affinity to BMPR-IB in comparison to ActR-IA and BMPR-IA (Ebisawa et al., 1999). Despite the complexity in the BMPR-ligand interactions, numerous research studies have identified a preferential of certain BMP ligands to bind to BMP type I receptors. For instance, BMP-2, BMP-4 and GDF-5 have a preference to bind to BMPR-IA and BMPR-IB (Aoki et al., 2001). Moreover, BMP-6 and BMP-7 preferentially bind to ActR-IA and BMPR-IB (Aoki et al., 2001). In addition, BMP-15 most readily binds to BMPR-IB and BMPR-IA, with a higher affinity to BMPR-IB (Moore et al., 2003). GDF-9 has been identified to bind to BMPR-II receptor (Vitt et al., 2002).

## **2.2 The BMPs**

The term BMP, first described in 1965 by Urist (Urist M., 1965), was used to describe bone components capable of stimulating bone and cartilage formation and repair (Shimasaki et al., 2004). In addition to mitogen stimulating activity, BMPs can also function uniquely as a morphogen in transforming connective tissue cells into osteoprogenitor cells (Rengachary, 2002, Yanagita, 2009). BMP ligands exist in the cell's cytoplasm as dimeric inactive pro-proteins containing a signal peptide, pro-peptide and mature portion. Proteolytic cleavage of the pro-peptide leads to the releases of mature peptide which forms homo or hetero dimers. BMP heterodimers have greater biological activity than their respective homodimers (Suzuki et al., 1997). Signal transduction by the BMP ligand requires heterodimers complex containing Type-I and Type-II trans-membrane serine-threonine kinase receptors (Liu et al., 1995). Mature BMPs are released into the extra-cellular compartment, where they bind to specific BMP cell membrane receptors (Degnin et al., 2004). BMPs are engaged in multiple cellular activities such as proliferation, cellular differentiation, migration, organization and apoptosis (Yanagita, 2009, Rossi et al., 2015).

### 2.4.1 Members of BMP family

The BMPs comprise of 20 members, of which seven were discovered originally. They are further subdivided into three categories according to their sequence identities. The first category contains BMP-2 and 4, the second category contains BMP-5, BMP-6 and BMP-7 (also known as osteogenic protein-1) while BMP-3 (also known as osteogenin) stands alone in the third category. Six of the seven BMPs, which were originally discovered (BMP 2-7), belong to TGF- $\beta$  superfamily while BMP-1 is a metalloprotease protein. Knockout analysis of one or more members of BMP family indicated redundant functions; however, each of them regulates specific functions which are summarized in **(Table 2-2)**.

**Table 2-2: Functional analysis of BMP genes**

*Sourced from: (Wozney, 2002)*

BMP Gene	Phenotype of Null Mutation in Mice
BMP-2	Lethal before formation of the skeleton. Defects in amnion, chorion and cardiac development
BMP-4	Early embryonic lethal with defects in development of the mesoderm
BMP-5	Mouse short ear locus. Mice are viable with defects in growth and patterning of skeletal elements. Also have impaired fracture repair
BMP-6	Viable mice with skeletal elements indistinguishable from wild type mice, slight delay in ossification of the sternum.
BMP-8	Duplicated genes (BMP8a and BMP8b) in mice; no skeletal phenotype; defects in spermatogenesis or placental development.
BMP-3	Viable with increased trabecular bone density. no other skeletal phenotype.
GDF-5	Mouse brachypodism locus. Alteration in number and length of skeletal elements of the limbs; joint formation defect.

#### **2.4.1 BMP-1**

Though BMP-1 was initially classified as member of BMPs family due to its stimulatory properties in bone and cartilage development, it was later discovered to also function as a metalloproteases with similarity to pro-collagen C proteinase (PCP) (Kessler et al., 1996). BMP-1 neither shares any structural similarity to other BMPs nor any growth factors; therefore, it is not categorized as a member of the TGF- $\beta$  superfamily (Shimasaki et al., 2004, Wozney et al., 1988). It is, rather, required to cleave the C-terminus pro-peptides of procollagen I, II and III during cartilage and bone development. BMP-1 is also involved in skin development in several species (Amano et al., 2000).

A study using immunohistochemistry technique has revealed that BMP-1 is expressed in the oocyte and the granulosa cells of all stages of follicle development in sheep suggesting that BMP-1 is involved in the follicle development (Canty-Laird et al., 2010), but the mechanisms is poorly understood.

#### **2.4.2 BMP-2 (BMP-2A or BMP-2 $\alpha$ )**

BMP-2 has been shown to be involved in diverse cellular physiological functions including development of cartilage (Wozney et al., 1988), bone (Tabas et al., 1991), differentiation of cardiac cell, transition from epithelial to mesenchymal type and signalling pathways, such as the hedgehog pathway, TGF  $\beta$  pathway and cytokine-cytokine interactions. BMP-2 appears to be critical for the embryological development, non-small cell lung carcinoma and tumor growth and osteoblast differentiation (Langenfeld et al., 2003). For instance, BMP-2 deficient mice were found to be incapable of surviving during embryogenesis (Zhang and Bradley, 1996). Finally, BMP-2 has been shown to augment FSH-induced estradiol production, FSHR expression in granulosa cells as well as Inhibin (A) production (Guangqin et al., 2013, Souza et al., 2002).

#### **2.4.3 BMP-4 (BMP-2B or BMP-2 $\beta$ )**

BMP-4 is a highly conserved regulatory protein of TGF- $\beta$  superfamily, which plays a vital role in the central and peripheral nervous system as well as in the development of the muscular and skeletal systems (Sedohara et al., 2002), specifically bones from mesoderm, cartilages, tooth and limb repair after fracture. BMP-4 along with fibroblast growth factor FGF2 stimulates stem cell differentiation to mesodermal lineages. Mice that have had the BMP-4 gene deleted, formed little or no mesoderm and died during gastrulation (Winnier et al.,



1995). In addition, BMP-4 heterozygous mice exhibited a range of birth defects such as a decreased number of primordial germ cells (PGC), polydactyl, abnormal kidney, eye and craniofacial development (Chang et al., 2002). In the Human ovarian theca-tumor (HOTT) cell culture model showed that BMP-4 treatment resulted in the reduction of forskolin-enhanced HOTT cells secretion of androstenedione, 17- $\alpha$ -hydroxyprogesterone and augmented production of progesterone (Dooley et al., 2000). BMP-4 is primarily expressed in theca cells of rat ovaries (Shimasaki et al., 2004). BMP-4 plays a bio-vital role in the reduction of FSH-induced production of progesterone and enhanced FSH-induced estradiol generation in rat granulosa cell cultures (Shimasaki et al., 2004). Furthermore, research in ewe pituitary by Faure et al. showed that BMP-4 reduced the secretion of FSH by stimulating the phosphorylation of Smad-1 (Faure et al., 2005). Thus, the effect of BMP-4 on FSH secretion was suggested to be due to the activation of BMP signalling pathways (Faure et al., 2005).

#### **2.4.4 BMP-5**

BMP-5 expression is associated with limb and bone, cartilage development, dendritic growth in the sympathetic nervous system as well as in cell death (Zuzarte-Luis et al., 2004). Its expression was observed in the trabecular meshwork, optic nerve head, lung and liver. BMP-5 does not show strong osteogenic potential in pre-osteoblast cell lines (Cheng et al., 2003). Several loss of function mutations showing short ear phenotypes and localized skeletal defects in mice have been assigned to BMP-5 gene function (Kingsley et al., 1992). Moreover, the expression of BMP-5 shown to associated with granulosa cell proliferation and steroid production in rat ovary (Faure et al., 2005).

#### **2.4.5 BMP-6 (VGR-1)**

BMP-6 was identified due to the ability of the demineralized bone extracts to induce endochondral osteogenesis in an extra-skeletal site. It plays a vital role in cartilage and bone development and known for inducing the osteogenic markers in mesenchymal stem cells (Yang et al., 2003). BMP-6, does share redundant functions with other BMP members, namely BMP 5 and 7. Single knockouts containing BMP-7 are either embryonic lethal to embryos or have severe phenotypic effects but unlike the double knockout (e.g of 5 and 6), single knockout of BMP-5 and 6 genes do not show gross abnormalities (Solloway and Robertson, 1999). The expression property of BMP-6 is more akin to BMP-2 in hypertrophic cartilage, suggesting that BMP-2 may be compensating for the functions of BMP-6 in homozygous mice

mutants (Solloway and Robertson, 1999). BMP-6 treatment of L- $\beta$ -T2 cell cultures in mice was associated with an increased production of endogenous FSH (Huang et al., 2001) by inhibiting adenylate cyclase (and therefore cAMP generation). This effect was seen in both responses to FSH action and to the adenylate cyclase activator Forskolin (Shimasaki et al., 2004). In context of ovarian function, BMP-6 has been demonstrated to inhibit some of the effects of FSH on gene expression, for example reducing the expression of FSH stimulated LH-receptor mRNA expression in follicle cells (Shimasaki et al., 2004). BMP-6 was also shown to be effective in blocking FSH-induced progesterone production, but interestingly not FSH-induced estradiol production (Shimasaki et al., 2004). During rat follicle development, BMP-6 mRNA expression was drastically reduced at the time of dominant follicle selection. Hence, it is suggested that the mechanism of dominant follicle selection prior to ovulation could be driven by downregulation of BMP-6 thus enhancing the FSH effect (Shimasaki et al., 2004).

#### **2.4.6 BMP-7 (OP-1)**

BMP-7 was initially purified as an osteogenic protein with a role in bone development, kidney, eye functions and reproduction (Lee et al., 2004). It has been shown that BMP-7 is co-expressed with BMP-5 in several tissues (Solloway and Robertson, 1999). Compound BMP-5 and BMP-7 mutant mice show cardiac (e.g. defective chamber septation) and other developmental abnormalities that are ultimately lethal. This indicates the function of BMP-5 in mutant mice was functionally compensated by BMP-7 which reflected the redundant nature of BMP-5 and BMP-7 previously mentioned (Solloway and Robertson, 1999). BMP-7 was shown to amplify FSH induced expression of P450 aromatase while blocking the mRNA expression of steroidogenic acute regulatory protein (StAR) (Lee et al., 2004). The study conducted by Lee W. et al. showed that BMP-7 modulates FSH-dependent estradiol and progesterone production by the expression of P450 arom and StAR (Lee et al., 2001b). BMP-7 was shown to reduce the number of primordial follicles, yet increase the number of primary, secondary as well as antral follicles, thus could enhance the recruitment of primordial follicles to the growing follicle pool (Shimasaki et al., 2004).

#### **2.4.7 BMP-15 (GDF-9B)**

BMP-15, an oocyte derived growth factor, also known as GDF-9B due to its high homology to GDF-9 and the co-expression with GDF-9 in oocytes (Yang et al., 2003). BMP-15 expression in oocytes is initiated during the recruitment of primordial follicles to primary follicles, and

expression continues until ovulation (Dube et al., 1998). Several mouse model analyses indicated that BMP-15 and GDF-9 play major role in late staged of folliculogenesis and following ovulation, however the functions of these two follow diverse paths in mono and poly-ovulatory mammals (Galloway et al., 2002). Genetic analysis studies have illustrated that heterozygous mutations in BMP-15 expression has resulted in the exhibition of hypergonadotropic ovarian failure, an under-developed uterus, primary amenorrhea and hirsutism (Di Pasquale et al., 2004). BMP-15 mitotic activity takes place independently from FSH, it can induces granulosa cell mitosis in pre-antral follicles, during the early stages of follicle growth (Shimasaki et al., 2004). similar to BMP-6, BMP-15, inhibits FSH-stimulated progesterone production without affecting the estradiol production in rat granulosa cells (Shimasaki et al., 2004). Other FSH induced actions such as LH receptor inhibin/activin expression and steroidogenesis are also blocked by BMP-15 (Shimasaki et al., 2004).

Follistatin, an activin binding protein, has been shown to play a crucial role in regulating folliculogenesis by binding to BMP-15, and inhibiting BMP-15 stimulation of granulosa cell proliferation, FSH receptor expression as well as progesterone generation (Otsuka et al., 2011, Shimasaki, 2006). Follistatin has been shown to be highly expressed in dominant follicles and minimally or untraceable in atretic follicles in rat ovaries.

#### **2.4.8 GDF-5**

GDF-5 is a member of the TGF- $\beta$  superfamily that is implicated in several skeletal developmental processes including chondrogenesis and joint formation. Mutations in the GDF-5 gene resulted in chondrodysplasias such as Hunter-Thompson and Brachydactyly (Nickel et al., 2005). GDF-5 exhibited a higher binding specificity to the type I receptor, exclusively binding with BMPR-IB (Mueller and Nickel, 2012). Deletion of either GDF-5 or BMPR-IB in mice exhibited a similar phenotype in both groups of mice, suggesting that GDF-5 signalling is greatly dependent upon the binding to BMPR-IB (Nickel et al., 2005). Human GDF-5 showed modulatory activity in regeneration of collagenous soft connective tissue and ligament (Jenner et al., 2007). Loss-of –function of GDF-5 [brachypodism] showed its importance for normal function specific set of skeletal elements (Storm et al., 1994). Structural as well as mutational analyses identified a single residue (Arg57) in the pre-helix loop of GDF-5 which is critical for high affinity and binding specificity to the BMPR-IB. variant

GDF-5 (GDF-5R57A) is capable of binding to both BMPR-IA and BMPR-IB with a higher affinity compared to the non-variant GDF-5 (Nickel et al., 2005).

#### **2.4.9 GDF-9**

GDF-9, a derivative of oocyte growth factor, controls numerous steps in folliculogenesis, including differentiation of all follicle compartments such as oocyte, follicle granulosa and thecal cells (Vitt et al., 2000). Whereas, GDF-9 was shown to be not required for male fertility, homozygous GDF-9 females were infertile. Adding GDF-9 to cultured human ovarian tissue promotes the early stages of folliculogenesis (Hreinsson et al., 2002). The growth and progression of the oocyte in GDF-9 deficient ovaries is abnormal showing a) faster development than control oocytes b) peri-nuclear organelle aggregation and abnormal peripheral Golgi complexes c) failure to develop cortical granules (Shimasaki et al., 2004). GDF-9 was shown to induce granulosa cell mitosis, where it increased granulosa cell proliferation and DNA production (Vitt et al., 2000). GDF-9 knockout mice ovaries lack the ability to recruit theca cell precursors, thereby lacking the expression of mRNA that codes for LH receptor in the thecal compartment (Elvin et al., 2000). GDF-9 increased Inhibin- $\alpha$  subunit production in neonatal ovaries (Hayashi et al., 1999) and stimulated Inhibin A and B generation by granulosa cells (Roh et al., 2003).

Also, GDF-9 was shown to be the inducer of progesterone synthesis, where GDF-9 promoted progesterone production in differentiated granulosa cells (Vitt et al., 2000, Vitt et al., 2001). Study conducted by Yamamoto, et al. on human granulosa cells showed that GDF-9 blocked progesterone production induced by cAMP and thereby indicating the variation in the mechanism GDF-9 between rat (Vitt et al., 2000) and human (Yamamoto et al., 2002) granulosa cells.

GDF-9 gene expression is predominantly located in the oocytes of neonatal as well as mature mouse ovaries (McGrath et al., 1995). Human GDF-9 shared 96% and 77% homology with mouse and ovine GDF-9 respectively (Dube et al., 1998, Bodensteiner et al., 1999). Oocytes from female mice with GDF-9 gene deletions show faster growth; however, the follicle growth is arrested at primary follicle stage (Carabatsos et al., 1998). Granulosa cells from knockout female mice also lose their mitotic capacity at later stages of the primary stage (Shimasaki et al., 2004).

**Table 2-3: Knockout phenotypes of phenotypes of several members of the BMP Signalling pathway**

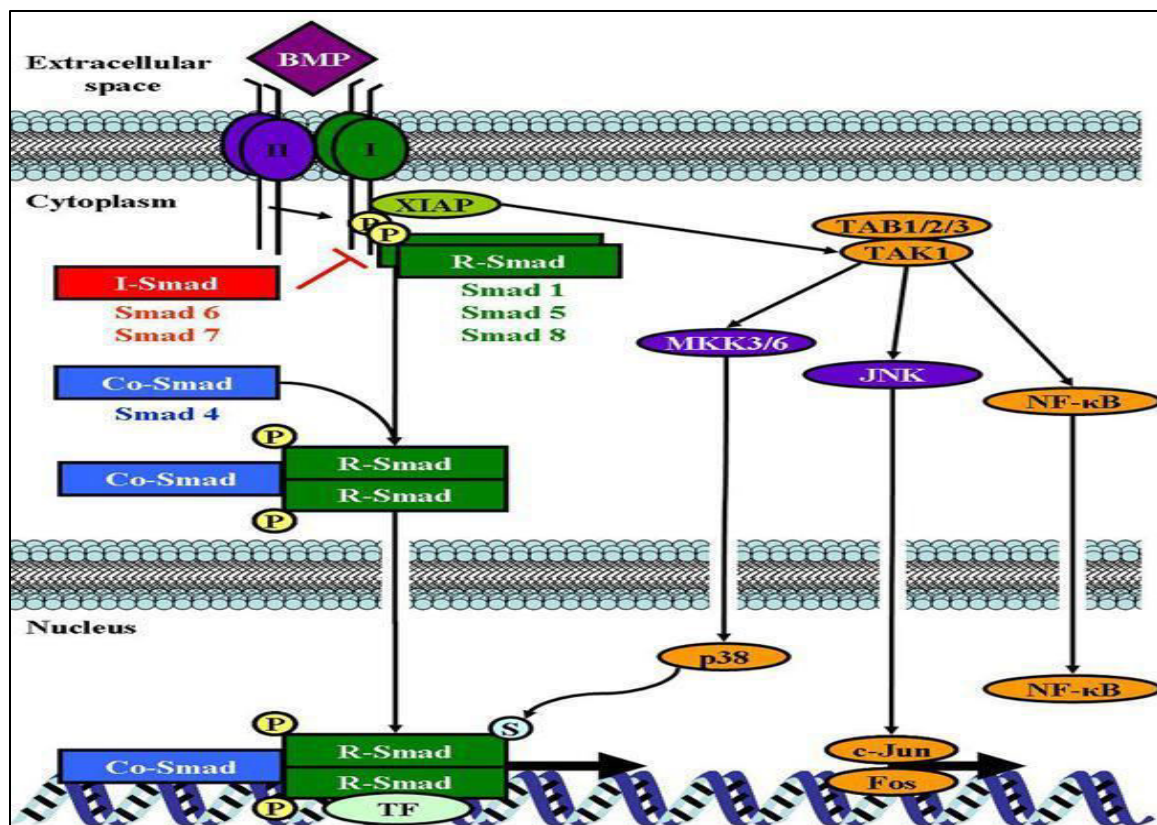
*Sourced from (Beederman et al., 2013)*

Deleted Gene	Cells affected	Skeletal phenotype	Reference
BMP2;BMP2+4	Chondrocytes	Chondrodysplasia, disorganised chondrocytes in growth plate, defects in chondrocytes proliferation, differentiation, increased apoptosis	<i>Shu et al. 2001</i>
BMP2+4	Limb bud	Failure of chondrogenic condensation to form normally, no formation of bone mesenchymal cells marrow cavity, trabecular or cortical bone	<i>Bandyopad-hyay et al, 2006</i>
BMP3	All cells	Increased bone density	<i>Daluiski et al, 2001</i>
BMP5	All cells	Short ear, brachypodism	<i>King et al, 1994</i>
BMP6	All cells	Minor sternal defects	<i>Solloway et al, 1998</i>
BMP7	All cells	Hindlimb polydactyly, defects in rib cage, skull	<i>Luo et al, 1995</i>
BMP11	All cells	Anterior-posterior axial skeletal patterning defects	<i>McPherron et al, 1999</i>
Alk2	Neural crest cells	Craniofacial malformations, including cleft palate, hypo-trophic mandible, reduced ossification of frontal bone	<i>Dudas et al, 2004</i>
BMPR1A	Osteoblasts	Increased bone mass, decreased bone resorption, reduced osteoclastogenesis	<i>Kamiya et al, 2008</i>
BMPR1B	Osteoblasts	Decreased bone mineral density, bone volume and bone formation	<i>Zhao et al, 2002</i>
Smad6	All cells	Axial and appendicular skeletal defects, posterior transformation of the cervical vertebrae, bilateral ossification centres in lumbar vertebrae, incomplete sternal band fusion	<i>Estrada et al, 2011</i>
Smad1	Chondrocytes	Delayed calvarial bone development	<i>Wang et al, 2011</i>
Smad1	Osteoblasts	Osteopenia, impaired osteoblast proliferation and differentiation	<i>Wang et al, 2011</i>
Noggin	Osteoblasts	Decreased weight, shortened femoral length, osteopenia	<i>Canalis et al, 2012</i>

## 2.3 BMP Signal Transduction

When a BMP ligand binds to the BMP receptor, the type II receptor trans-phosphorylates the type I receptor at an intracellular juxtamembrane site rich in glycine and serine residues named the GS domain (Wrana et al., 1992). Consequently, the type I receptor Trans-phosphorylates the R-Smads signalling proteins: Smad 1,2,3,5 and 8 (Ten Dijke et al., 2002). The activated R-Smad proteins bind together in multiple protein complexes with Smad-4 (also termed as the common mediator Smad or Co-Smad) (Nishimura et al., 1998, Massague et al., 2005). The R-Smad–Co-Smad complex then moves to the nucleus. Inside the nucleus these complexes, which then bind to specific areas of DNA and collaborate with particular

transcription factors where they regulate the activity of target genes and thus control cell responses such as proliferation (Shimasaki et al., 2004). There are significant differences in the Smad signalling pathways of the BMPs and that of activins/TGF- $\beta$ s (Miyazawa et al., 2002). Activins and TGF- $\beta$ s regulate the intracellular signals through the phosphorylation of Smad-2 and Smad-3 whereas almost all BMP (BMP-2, BMP-4, BMP-6, BMP-7 and BMP-15; GDF-5 and MIS) ligands mediate their intracellular signals through the phosphorylation of Smad-1, Smad-5 and/or Smad-8 (Shimasaki et al., 2004). **Figure 2-2** provides a detailed description of the BMP signal transduction pathway. Further research illustrated that both BMPs as well as Activins can bind to ActR-II and ActR-IIB, hence the major determinant of Smad signalling is dependent on the specificity to binding to type-I receptors (Attisano and Wrana, 2002).



**Figure 2-2: BMP signal transduction pathway.**

Sourced from: (Marquis et al., 2009)

Further validation of the studies has shown that high expression of active forms of BMP-type I receptors leads to the activation of Smad-1, Smad-5 and Smad-8, and induction of BMP-specific biological responses (Chen et al., 1997). On the other hand, GDF-9 was shown to

phosphorylate Smad-2 rather than Smad-1/5/8 in human granulosa cells (Kaivo-Oja et al., 2003).

## **2.4 Factors affecting BMP signaling**

BMP signalling is controlled and regulated at various levels by intracellular as well as extracellular factors (Rossi et al., 2015). The factors that alter and modulate BMP signalling include the Inhibitory Smads that block BMP signalling, the BMP binding proteins that inhibit BMPs from binding to receptor and many other factors that utilize different mechanisms (Massague et al., 2005).

### **2.4.1 Intracellular regulatory Factors**

One of the major regulatory intracellular factors of BMP signalling is the transmembrane glycoprotein BAMBI (BMP & Activin Membrane Bound Inhibitor), which is expressed in the granulosa as well as the theca cells of the ovary (Luo et al., 2012, Loveland et al., 2003, Onichtchouk et al., 1999). The extracellular domain of BAMBI is similar to that of type I receptors of BMPs, which could compete for various BMP ligands. However, unlike the BMPR, the intracellular kinase domain is absent (Onichtchouk et al., 1999). The absence of such intracellular kinase domain of BAMBI fails to form receptor complexes necessary for further signalling events (Onichtchouk et al., 1999) hence negatively modulates the BMP signalling. Thus, BAMBI can bind to the ligands and block the signalling of BMPs to act as a dominant negative receptor (Grotewold et al., 2001).

The Ski and Tob proteins are other intracellular regulatory factors of BMP signalling in both *in vivo* and *in vitro* (Wang et al., 2000). The Ski and Tob proteins inhibit BMP signalling by binding to the MH<sub>2</sub> domain of Smads (Yoshida et al., 2000). Nonetheless, the Ski and Tob proteins may not be involved in reproduction, since the Ski and Tob knockout mice exhibited no phenotypic defects associated to reproduction (Berk et al., 1997).

The Smad Ubiquitination Regulatory Protein-1 (Smurf 1) is an intracellular factor that negatively regulates BMP signalling by interacting with R-Smads to block BMP signal transduction (Zhang et al., 2001). Smurf 1 inhibits BMP signalling by blocking the Smad 1 and Smad 5, thereby, leading to their proteosomal destruction (Zhu et al., 1999). Smurf 1 was shown to reduce the intracellular levels of Smads, independent of BMP ligand activation pathways (Zhu et al., 1999).

#### **2.4.2 Extracellular regulatory Factors**

BMP signalling is also regulated extracellularly by various BMP binding proteins and BMP ligands. These proteins inhibit BMP signalling by blocking the access of ligands to their respective receptors (Gamer et al., 2005). Follistatin, Gremlin and Noggin are several such examples of extracellular proteins that inhibit BMP signalling (Ali and Brazil, 2014). BMP signalling may also be modulated by the MAPK family of signalling molecules such as ERK 1/2, p38 and stress-activated protein kinase/Jun N-terminal kinase (Yamaguchi et al., 1995, Nakamura et al., 1999). MAPK signalling molecules were shown to act both as stimulators of BMP signalling (Gallea et al., 2001) or inhibitors of Smad signalling pathways (Kretzschmar et al., 1997).

Follistatin, extracted from bovine and porcine ovarian follicle fluid, is another extracellular factor that plays a vital role in BMP signalling (Iemura et al., 1998). Follistatin expression was observed in nearly all human tissues predominantly in adult ovary, pituitary gland, kidney, fetal heart and liver (Tortoriello et al., 2001). Follistatin is capable of binding to several members of the TGF- $\beta$  superfamily, including BMPs (Iemura et al., 1998) such as BMP-4 (Yamaguchi et al., 1995), BMP-7 (Iemura et al., 1998) and BMP-15 (Otsuka et al., 2011). However, it had antagonizing effect on the BMP-2 induced osteoblast differentiation (Abe et al., 2004). In addition, female mice overexpressing follistatin showed smaller size ovaries due to the inhibitory function in folliculogenesis (Guo et al., 1998).

Inhibin, a member of the TGF- $\beta$  superfamily, is another extracellular factor that regulates BMP signalling (Shimasaki et al., 2004). Inhibin blocks the FSH secretion in anterior pituitary cell cultures from human follicle fluids (Chari et al., 1979). It was hypothesized that Inhibins act as competitive inhibitor with the BMPs for the binding to activin type II receptors for inhibition of BMPs induced responses (Wiater and Vale, 2003). Thus, Inhibin was shown to block the actions of both BMP-2 and BMP-7 (Wiater and Vale, 2003).

Gremlin encodes extracellular factor that modulates BMP signalling by selectively inhibiting the BMP-4 related functions (Shi et al., 2001). Gremlin contained a structural feature that is very similar to the cysteine knot present in the TGF- $\beta$  superfamily (Hsu et al., 1998).

Noggin encodes an extracellular factor with BMP antagonistic properties (Smith and Harland, 1992). Noggin was first discovered for its regulatory function in normal dorsal development in *Xenopus* embryos (Smith and Harland, 1992). Noggin blocks BMP signalling by obstructing the binding of type I/type II receptors of BMPs (Groppe et al., 2002). Further researches have



shown that Noggin acts as an inhibitory protein for BMP-2, -4, -7, -14 and GDF-5 (Shimasaki et al., 2004).

## **2.5 The Regulatory role of BMPs in the Ovary**

The impact of the BMPs family on the ovarian function has been demonstrated by using different experimental approaches in different animal species. For instance, the administration of BMP-7 by Lee *et al.* to the ovarian bursa of a female rat led to a considerable reduction in the number of primordial follicles and a significant increase in the number of primary, pre-antral and antral follicles (Lee et al., 2001b). Thus, BMP-7 inhibits the primordial follicle formation, however, facilitates follicle development at primary, pre-antral and antral follicles in the follicle cycles. In addition, the administration of BMP-7 to rat granulosa cells amplified DNA synthesis as well as granulosa cell proliferation (Lee et al., 2001b).

In a separate study, Vitt et al in 2000 observed enlargement of the ovaries and a significant increase in primary and small pre-antral follicles and a reduction in the number of primordial follicles due to the administration of GDF-9 to the ovaries of immature rats for several days (Similar results were obtained by Shimizu T. *et al.* (2004) after injecting GDF-9 gene fragments to the ovaries of 2-month old pre-pubertal gilts (Shimizu et al., 2004, Vitt et al., 2000).

In mice, it is well established that primordial germ cell (PGC) determination is induced by the extra-embryonic ectoderm in epiblast cells (Saitou et al., 2002). Both BMP-4 as well as BMP-8b was shown to play vital roles in PGC formation in mouse embryo (Shimizu et al., 2004). The genes encoding BMP-4 and BMP-8b were co-expressed in the extraembryonic ectoderm prior to the detection of PGCs (Ying et al., 2001). Furthermore, BMP-4 knockout mice were embryo-lethal during early gastrulation; however, mice which survived gastrulation thrived long enough with no later development of the PGCs (Lawson et al., 1999). However, the regulatory details of involvement of BMP-4 expression for PGCs are yet to be known.

Thus, summarily BMP-4 is responsible for the formation of primordial follicles during embryonic development. In addition, BMP-4 increase the number of primordial follicles developing into primary follicles consequently reduces the number of primordial follicles (Nilsson and Skinner, 2003). Also, it reduces the size of the ovaries in whole ovary culture as a result of the loss of oocytes, primordial follicles and cellular apoptosis (Nilsson and Skinner, 2003).

In a study on rat Graafian follicles by Erickson and Shimasaki 2003, the oocyte has been shown to express BMP-6, BMP-15, GDF-9, ActR-1A, ActR-II, BMPR-IA and BMPR-IB, the granulosa cells showed expression of BMP-2, BMPR-IA, BMPR-IB and BMPR-II, and theca cells showed expression of GDF-10, BMP-4, BMP-7, BMPR-IA and BMPR-IB (Erickson and Shimasaki, 2003). The BMP-6 mRNA transcripts were shown to be absent in the granulosa cells of the rat primordial and primary follicles, which was demonstrated in the dominant follicle (Erickson and Shimasaki, 2003). From this result it was concluded that the elimination of BMP-6 expression was the trigger for dominant follicle selection in the rat (Shimasaki et al., 2004). Studies on experimental mice have illustrated that oocyte derived growth factors act to influence FSH-dependant cyto-differentiation in granulosa cells (Otsuka et al., 2011). During the development of Graafian follicle, GDF-9 and BMP-15 produced by the oocyte, provide gradient signals to produce the different subtypes of granulosa cells, the influence being related to their location and distance relative to the oocyte. The different subtypes of granulosa cells generated play a critical role in ovulation (Williams and Erickson, 2008).

In sheep ovaries, BMPR-IA, BMPR-IB and BMPR-II were identified in the granulosa cells of primary, antral follicles, the late antral stage, ovarian surface epithelium as well as the corpus luteum (Souza et al., 2002). The inhibitory effects of BMP on progesterone production from granulosa cells diminished with follicle development (Fabre et al., 2003). Hence such inhibitory effects were greater on granulosa cells obtained from small antral follicles than from pre-ovulatory follicles (Fabre et al., 2003). On the other hand, activin and TGF- $\beta$ 1 had similar effects on progesterone production by granulosa cells regardless of the follicle stage of follicle development (Fabre et al., 2003). Thus, such an alteration accounted for the effects of the BMPR-IB in steroidogenesis, where both BMP-4 and GDF-5 interact with BMPR-IB for signalling (Fabre et al., 2003). BMP-4 was demonstrated to block basal and FSH-induced generation of progesterone from sheep granulosa cell cultures (Mulsant et al., 2001). Moreover, BMP-2 has been illustrated to enhance the production of estradiol from sheep granulosa cells (Souza et al., 2002).

In rat granulosa cell cultures, BMP-7 caused the increase of FSH-induced estradiol production, blocked FSH-induced progesterone production (Shimasaki et al., 2004) and enhanced DNA synthesis (Lee et al., 2001b). Such effects of BMP-7 on estradiol and progesterone production could be the consequence of its effect on P-450 aromatase (converts androstenedione to

estradiol) and STAR (transports cholesterol across the mitochondrial membrane) (Lee et al., 2001b).

BMP-5 was shown to block basal and FSH induced production of progesterone in rat granulosa cell cultures (Pierre et al., 2005) but had no effect on estradiol production (Pierre et al., 2005). Such effect of BMP-5 on progesterone production was associated with reduced expression of STAR (Pierre et al., 2005). BMP-5 also caused enhanced cyclin D2 expression (protein engaged in cell proliferation and differentiation) leading to the production of granulosa cells (Pierre et al., 2005). Knockout of Cyclin D2 in female rats resulted in infertility because of their granulosa cells being unable to proliferate in the presence of FSH (Sicinski et al., 1996).

BMP-15 was shown to enhance the proliferation and differentiation of granulosa cells, independent of FSH induction in rat granulosa cell cultures (Otsuka et al., 2011). BMP-15 reduces FSH induced progesterone production without affecting the estradiol production (Otsuka et al., 2001). Also, reduction of FSH induced expressions of STAR, (LH-R) and (FSH-R) by BMP-15 indicated that BMP-15 exerts its effects through up-regulation of cAMP signalling (Otsuka et al., 2001). Therefore, BMP-15 reduces both basal and FSH induced expression of FSH-R in rat granulosa cell cultures (Otsuka et al., 2001). Hence, BMP-15 could act as a vital factor for modulating FSH action by direct action on FSH-R or indirectly by exhibiting its effect on different factors such as Follistatin, Inhibin and Activin (Otsuka et al., 2001). **Table 2-4** shows the effects of various members of the TGF- $\beta$  superfamily, in terms of mRNA expression, in different stages of folliculogenesis (Erickson and Shimasaki, 2003). BMP-6, BMP-15 and GDF-9 were expressed in the oocyte of different follicles (Erickson and Shimasaki, 2003). For instance, the expression of both GDF-9 and BMP-15 in the follicle fluid illustrated that they were secreted products (McNatty et al., 2006). GDF-9 expression was observed in the oocyte of primary and secondary follicles in rats (Jaatinen et al., 1999) and humans (Aaltonen et al., 1999), whereas in bovine ovaries, GDF-9 was also expressed in the oocyte of primordial follicles (Bodensteiner et al., 1999).

**Table 2-4: The secretory activity of different follicle cell types (m-RNA) of theca cells, granulosa cells and oocyte- and its relation to intra-ovarian signaling molecules, including the BMP family**

*Sourced from: (Erickson and Shimasaki, 2003)*

TISSUE	BMP-2	BMP-3	BMP-3b	BMP-4	BMP-6	BMP-7	BMP-15	BMPR-IA	BMPR-IB	BMPR-II	FS
<b>Oocyte</b>											
Primordial	-	-	-	-	-	-	-	+	++/+	+/-	-
Primary	-	-	-	-	-	-	+	++	+++/>++	+	-
Secondary	-	-	-	-	+++/>+	-	++++/>++	+++	++++	+/-	-
Tertiary	-	-	-	-	+++	-	++++	+++	+++	+/-	-
Dominant	-	-	-	-	+++	-	++++/>+++	++	+++	+/-	-
Atretic	-	-	-	-	+++/>++	-	++/>+	+++/>++	++	+/-	-
<b>Granulosa Cells</b>											
Primordial	-	-	-	-	-	-	-	+/-	+/-	-	-
Primary	+/-	-	-	-	-	-	-	+	+/-	+	-
Secondary	++/>-	-	-	-	+++/>-	-	-	++/>+	++++	+++/>+++	++
Tertiary	+++/>-	-	-	-	+++/>-	-	-	++	++++	+++	++++
Dominant	+++/>-	-	-	-	+/-	-	-	++	++++	+++	++++
Atretic	++++	-	-	-	+++	-	-	++	++++	+++	+/-
<b>Theca-interstitial cells</b>											
Primordial	-	-	-	-	-	-	-	-	-	-	-
Primary	-	-	-	+	-	-	-	-	-	-	-
Secondary	+/-	-	-	++/>-	-	++/>-	-	+	+	-	-
Tertiary	+/-	+/-	+	+++/>-	-	+++/>-	-	++/>+	+	-	-
Dominant	+/-	+/-	++++/>-	++++/>-	-	+++/>-	-	++/>+	++	-	+/-
Atretic	+/-	-	+/-	+/-	-	+/-	-	+	+	-	-
<b>Theca externa</b>											
	-	-	++++/>-	++++/>-	-	-		+	++/>-	-	

In the study conducted by Juengel *et al* in 2006, BMP-6 mRNA but not BMP-2, BMP-4 or BMP-7 mRNA was expressed in the non-atretic sheep follicles (Juengel et al., 2006). BMP-6 expression was confined only to the oocyte of primordial, primary, pre-antral as well as antral follicles (Juengel et al., 2006). BMP-2, BMP-4, BMP-6 and BMP-7 each blocked progesterone synthesis in granulosa cells of sheep and rat. However, the cellular proliferation and growth of the granulosa cells was enhanced in case of the rat, whereas not affected in case of sheep indicating differential regulation and species-specific difference for these growth factors (Juengel et al., 2006).

Further studies by Juengel et al. showed that BMP-2, BMP-4 and BMP-7 did not play a role in regulating follicle development in sheep ovaries due to the lack of expression of BMP-2, BMP-4 and BMP-7 in the non-atretic ovarian follicle (Juengel et al., 2006). On the other hand, the presence of BMP-6 mRNA in the oocyte provides evidence that BMP-6 may have a paracrine

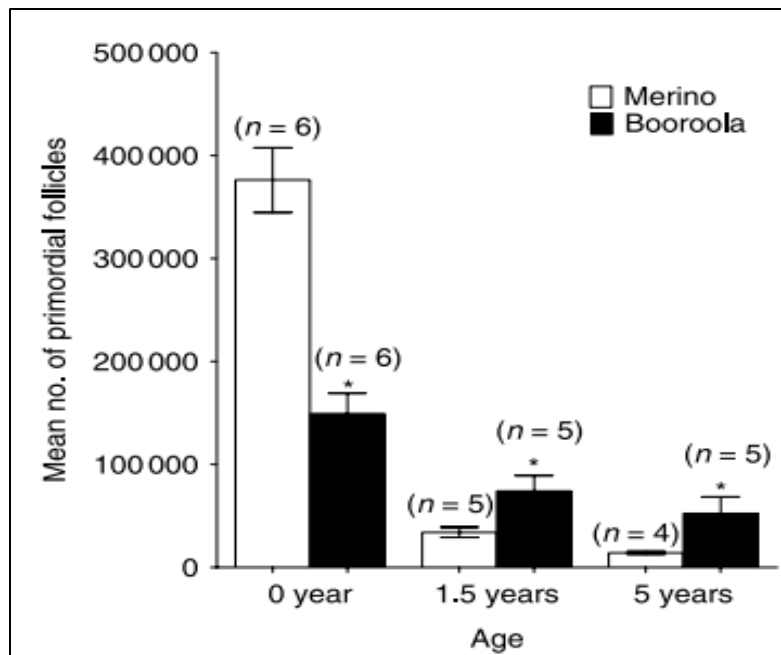
and/or autocrine role in regulating follicle development in sheep, similar to BMP-15 and GDF-9 (Juengel et al., 2006).

## 2.6 Sheep breeds with a high ovulation rate

Different breeds of sheep possess different ovulation rates. Breeds such as Romanov, *Booroola Merino*, Olkuskas, Belcare, Inverdale, Cambridge, Woodlands and Javanese are genetically different (Souza et al., 2004, Campbell et al., 2009, Ruoss et al., 2009, Juengel et al., 2013). The *Booroola Merino* ewe is the preferred breed of sheep for experimental analysis due to its distinctive characteristics (Bindon et al., 1985). A study conducted by Piper and Bindon (1982) postulated that the difference in ovulation rates between *Booroola* and *Merino* ewes could be contributed by a single major gene (Piper and Bindon, 1983). It was later discovered in segregation studies that the *Booroola* phenotype is due to a mutation in the autosomal gene called FecB (Montgomery et al., 1993).

Higher prolificacy and ovulation rates in homozygous *Booroola* ewes were shown to be related to FecB gene (Hua and Yang, 2009). Introduction of FecB gene to low prolific breeds of sheep clearly showed an improvement in the reproductive rate (Hua and Yang, 2009). Further research conducted by Wilson et al. on the *Booroola* phenotype discovered the mutation Q249R in the intracellular kinase signalling domain of BMPR-IB, which was responsible for the receptor specific for binding to BMP-4 and GDF-5 (Wilson et al., 2001). The point mutation Glutamine 294 Arginine in the intracellular kinase signalling domain converted the non-carrier ewes to in carrier ewes of *Booroola* phenotype was caused due to alteration in the encoded BMPR-IB protein (Wilson et al., 2001). The granulosa cells from *Booroola* ewes were less sensitive to BMP-4 and GDF-5 with regards to inhibition of progesterone production (Fabre et al., 2003). In other words, the mutation in the Q249R caused a reduction in the BMP-dependent functions of the BMPR-IB receptor (Shimasaki et al., 2004). Further research by Ruoss et al. on *Booroola* and *Merino* whole sheep ovaries at birth, 1.5 years and 5 years demonstrated that *Booroola* sheep ovaries at birth had a considerably lower number; however, at 1.5 years and 5 years showed a reversed pattern with a considerably higher number of primordial follicles than *Merino* sheep ovaries (Ruoss et al., 2009) (**Figure 2-3**). Moreover, the rate of primordial follicle recruitment was significantly lower in *Booroola* ewes compared to *Merino* ewes (Ruoss et al., 2009). As such, 51% and 66% of primordial follicle consumption took place in *Booroola* ewes at 1.5 and 5

years respectively compared to 92% and 97% of primordial follicle consumption in *Merino* ewes at 1.5 and 5 years (Ruoss et al., 2009).



**Figure 2-3: Number of primordial follicles in Booroola/Merino sheep at birth, 1.5 years and 5 years.**  
Sourced from: (Ruoss et al., 2009)

## 2.7 The impact of mutation of functional (BMPs) on female reproduction

Mice with targeted deletion of functional BMP genes were lethal prior to the development of the reproductive system (Zhao, 2003). For example, the deletions of BMP-2 and BMP-4 genes were embryonic lethal due to significant abnormalities in amnion/chorion/cardiac development (Zhang and Bradley, 1996) and the lack of mesoderm induction (Winnier et al., 1995) respectively. However, few mice with targeted deletions of BMP genes deleted remained viable albeit reproductive defects and abnormal fertility (Shimasaki et al., 2004). BMPR-IB expression was observed in the oocyte of maturing follicles, the oocyte and granulosa cells of antral follicles as well as in the uterine endometrium (Yi et al., 2001). On the other hand, BMPR-IB was absent in the granulosa cells of atretic follicles and in the pituitary gland (Yi et al., 2001). Thus, the absence of BMPR-IB in the pituitary gland indicated that BMPR-IB had no direct role in regulation of gonadotropin hormones production (Yi et al., 2001). In *Booroola* sheep, BMPR-IB mutation resulted in ovarian follicles undergoing premature maturation, thereby leading to three to seven follicles ovulating at smaller

diameters compared to their counterparts (*Marino* sheep) with normal BMPR-IB expression (McNatty et al., 2005).

Deletion of BMPR-IB gene resulted in infertility and disrupted estrous cycles without affecting ovarian follicular development in female mice (Yi et al., 2001). Moreover, the Cumulus Oocyte Complex (COC) of BMPR-IB knockout mice had less cumulus cells than the COC of control mice. These results indicated that the inability of BMPR-IB knockout mice to exhibit cumulus cell expansion was the reason for their infertility and infertility initiated after ovulation (Yi S. et al., 2001). The findings were supported by the fact that oocytes were capable of undergoing fertilization in vitro but not in vivo. Thus, the BMPR-IB mutation has potential implications to reduced ability to block the differentiation of follicular cells by the various BMPs (McNatty et al., 2005).

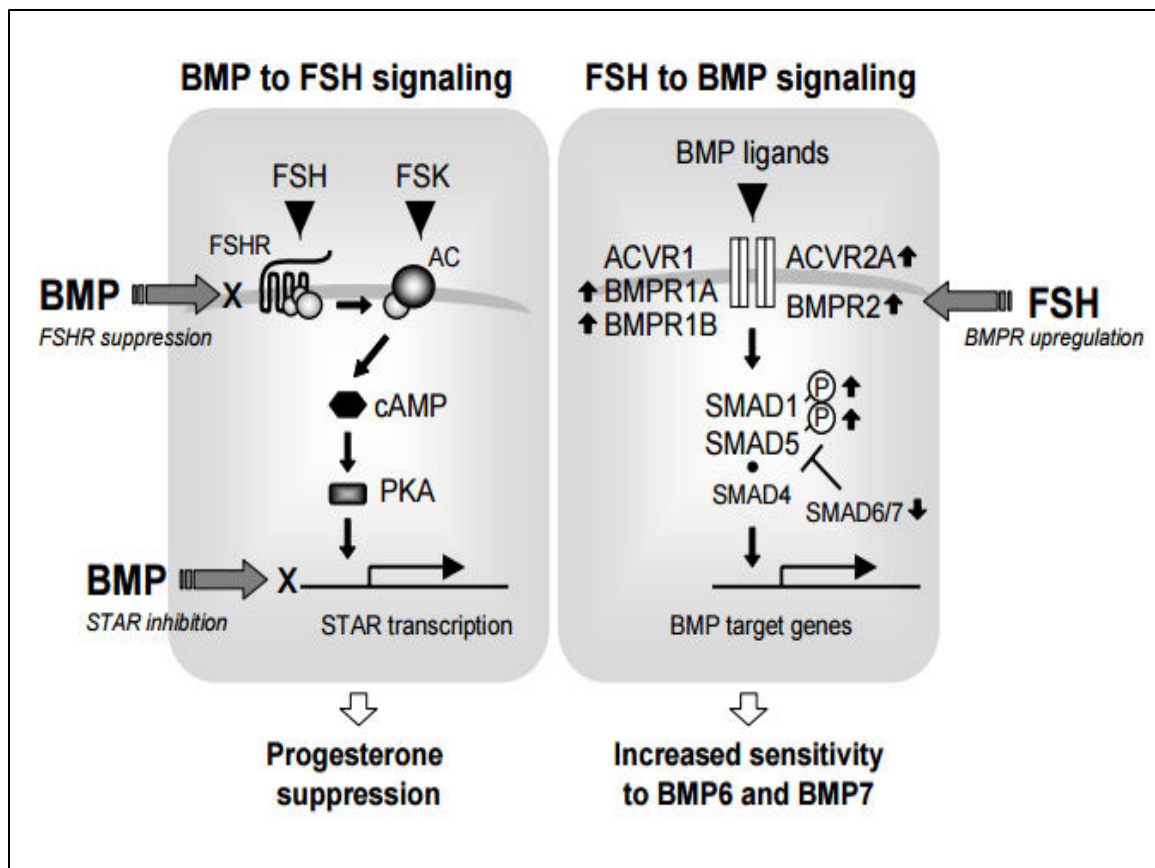
In sheep, the five point mutations in BMP-15 and one-point mutation in GDF-9 gene were believed to result reduced levels of mature protein and distorted binding to cell-surface receptors. Sheep heterozygous for the GDF-9 and/or BMP-15 mutations showed higher ovulation rates than their Wild-type counterparts. Conversely, sheep homozygous for the GDF-9 and/or BMP-15 mutations resulted infertility due to follicular growth / developmental arrest (Functional BMP-15 protein was present exclusively in sheep oocytes from the primary stage, whereas GDF-9 protein was found exclusively in oocytes throughout follicular development (McNatty et al., 2006).

A clinical case study by Demirhan et al. (2005) on a 16-year-old girl with homozygous mutation of BMPR-IB, encompassing deletion of the extracellular ligand binding domain of BMPR-IB, resulted in severe limb malformations, genital anomalies as well as primary amenorrhea. Ultra-sonographic investigation further revealed the absence of ovaries as well as a hypoplastic uterus. Further endocrinological examination revealed a hyper-gonadotrophic hypogonadism syndrome, indirectly indicating ovarian dysfunction in the patient (Demirhan et al., 2005).

## 2.8 The interplay between gonadotropin and BMP in folliculogenesis

BMPs play a vital role in mediating folliculogenesis by regulating the actions of FSH in the ovary (Miyoshi et al., 2006, Ruoss et al., 2009, Takeda et al., 2012). FSH was shown to induce BMPR-IA and BMPR-IB receptor expression, enhance expression of Smad-1 and Smad-5 proteins, as well as suppress the expression of the inhibitory Smads- Smad-6 and Smad-7 (Hosoya et al., 2015). Also, FSH was shown to augment BMP-induced Smad-1, Smad-5 and Smad-8 phosphorylation. Since overexpression of BMPR-IA and BMPR-IB considerably enhances BMP responses, such BMP type I receptors were considered to be the limiting factors for the BMP signalling pathway (Miyoshi et al., 2006, Takeda et al., 2012, Hosoya et al., 2015). BMP-4 and BMP-2 were shown to increase FSH-induced estradiol and reduce progesterone production in rat (Shimasaki et al., 2004) and sheep (Souza et al., 2002) granulosa cells respectively. In other studies, it was reported that BMPs block the normally gonadotropin induced progesterone production, brought by Forskolin without affecting the estradiol production (Miyoshi et al., 2006). BMPs could also modulate steroidogenesis downstream to cAMP formation, since cAMP levels induced by Forskolin were not modified by BMPs. The FSH-cAMP pathway was closely related and functionally linked to the BMP system, hence crucial for the maintenance and development of granulosa cell proliferation depends on the FSH-cAMP pathway, which is closely related and functionally linked to the BMP system (Guangqin et al., 2013, Castilho et al., 2014, Hosoya et al., 2015). Moreover, BMPs were shown to down regulate FSH receptors, thereby contributing to the maintenance of granulosa cell functions by regulating BMP ligands and FSH sensitivity (Wang and Roy, 2009). During follicle growth and development, the granulosa cells exhibited FSH receptor and proliferated upon FSH stimulation (Shi et al., 2010). LHR expression also increased in the pre-ovulation stage in the granulosa cells. Hence, the LHR increased the sensitivity of the granulosa cells to the LH surge (Shi et al., 2010). Specifically, BMPs down regulate FSH receptors expression, thereby contributing to the maintenance of granulosa cell functions by regulating the sensitivity to FSH (Wang and Roy, 2009). In contrast, BMP-2 was demonstrated to induce FSH receptor expression while reduce the StAR and LH receptor expression in human granulosa cells (Shi et al., 2011). **Figure 2-4** depicts the mutual regulation of BMP and FSH signalling in granulosa cells.





**Figure 2-4: Mutual regulation of the BMP system and FSH signaling in granulosa cells Sourced from (Miyoshi et al., 2006)**

Both BMP-6 (Shi et al., 2009) and BMP-7 (Shi et al., 2010) are expressed in theca and granulosa cells, and both increase FSH receptor and decrease LH receptor expression in granulosa cells. BMP-15, produced exclusively from the oocytes in the ovary, suppresses FSH activities by blocking FSH receptor expression (Otsuka et al., 2001b). Moreover, GDF-9 derived from the oocyte also blocks FSH-induced steroidogenesis as well as LH receptor expression in rat granulosa cells (Vitt et al., 2000b). Both BMP-15 and GDF-9 are expressed in the oocytes and mutations led to the abrupt halt of folliculogenesis in human (Laissue et al., 2006).

Chen et al. (2009) investigated the expression of BMP-15 and GDF-9 genes in sheep granulosa cells treated with FSH. Treatment of sheep granulosa cells with FSH (1-10 ng/ml) alone reduced the expression of BMPR-IB (Chen et al., 2009). While, the treatment of sheep granulosa cells with FSH (5 ng/ml) and estradiol (E2) (1 ng/ml) enhanced the expression of BMPR-IB. Consequently, the study suggested that the expression of BMPR-IB was modulated by FSH and E2 in sheep granulosa cells (Chen et al., 2009).

BMPs were illustrated to play a role in the regulation of FSH production and secretion in the pituitary gland (Young et al., 2008). Pituitary cells from *Booroola* ewes with natural mutation

in BMPR-IB secrete less FSH than wild type ewes, in the presence of BMP-2, BMP-4, BMP-6 and GDF-9 (Young et al., 2008). However, the BMPs did not influence the amount of FSH stored in the cells or the levels of secreted LH (Young et al., 2008). Furthermore, the BMPR-IB receptor was not found to co-exist with gonadotrophin cells in *Booroola*/Wild-type pituitary cell. Thus, the results of the study implicated that the effect of BMP-2, BMP-4, BMP-6 and GDF-9 does not occur under the direct action of the mutant BMPR-IB receptor in the cells that synthesize FSH (Young et al., 2008).

Despite the well documented evidence present to date of how the BMP system modulates FSH action in granulosa cells, a clearer understanding of how FSH modulates the BMP system in the ovary is yet to be determined. It remains clear though that FSH actions throughout folliculogenesis are strictly modulated by paracrine as well as autocrine factors as the follicles grow and develop (Webb et al., 2003).

# Three-Dimensional Image Analysis to Quantify the Temporo-Spatial Expression of Cellular Receptors

Sarmed Al-Samerria and Ghanim Almahbobi

School of Biomedical Sciences, CHIRI, Curtin University, Perth, WA, Australia

Email: sarmed.alsamerria@postgrad.curtin.edu.au; g.almahbobi@curtin.edu.au

**Abstract**—Ovarian folliculogenesis is primarily controlled by the action of gonadotropins namely follicle stimulating hormone (FSH) and luteinizing hormone (LH). Several reports indicated that the process of initial recruitment of primordial follicles to the growing follicles is not gonadotropin-dependent but Bone morphogenetic protein (BMP)-dependent. However, this has not been unequivocally confirmed. The aim of this study was to investigate the temporo-spatial protein expression of the BMP receptors 1B (BMPR1b), FSHR and LHR in several stages of follicle development. While the localization of all receptors was found in granulosa cell membrane of the follicles the temporal expression was varied. BMPR1b was expressed in all follicle stages, FSHR was detected in primary follicles onward and LHR was absent in both primordial and primary follicles but appeared in later stages. Quantitative analysis based on the intensity of fluorescent signals showed that the expression of BMPR1b, FSHR and LHR significantly ( $p < 0.001$   $p < 0.0001$   $p < 0.0001$  respectively) increased with follicular development. We have concluded that the combination of sensitive immunofluorescence labeling and computerized 3D image analysis proves efficient tools for in situ detection and quantification of the expression of small amount of protein in a complex tissue structure.

**Index Terms**—folliculogenesis, Bone morphogenetic protein, follicle stimulating hormone (FSH), luteinizing hormone (LH), 3D image analysis

## I. INTRODUCTION

Ovarian folliculogenesis is the basis of the entire ovarian function including the production of mature oocytes ready for fertilization and the sexual hormones required for the development of female phenotypes. This process entails a series of chronological steps in which a growing follicle either matures to ovulation or dies by apoptosis [1]. The primordial follicle considered as the constructional units of female ovary. The initial transition of primordial follicles to growing primary follicles is the key limiting step in preserving or depletion of the female fertility reserve. It is well established that folliculogenesis is regulated by the gonadotropins FSH and LH, which are secreted by the pituitary gland [2]. In addition, complex autocrine and paracrine actions of several intra-ovarian factors such as the BMPs influence the action of gonadotropins [3]. For instance, it is now believed that

the initial stage of follicle recruitment and development of primordial to primary follicles is independent of the gonadotropins [4] but rather associated with a specific spatial and temporal pattern of BMP expression [5].

However, such interplay between the gonadotropins and BMPs particularly in the initial recruitment of primordial

Follicles remain inconclusive. Because of the complex nature of the ovarian structure and the dynamics of folliculogenesis, most of the studies in relation to this issue have been conducted in vitro using molecular and biochemical approaches [6] or in situ microscopic analysis using manual subjective methods [7].

The use of new non-bias computerized technology in biomedical research has increased in the past decades. The computer-based method was introduced to provide accurate and reliable quantitative information as well as a non-time consuming tool [8]. This technology will eventually replace the conventional manual and subjective methods. In attempt to clarify the regulation of initial folliculogenesis; the present study demonstrates the interaction between BMP and gonadotropin signaling systems, using immunofluorescence detection and 3D image analysis of cell-membrane bound receptors of BMPs, FSH and LH in developing follicles of sheep ovaries.

## II. MATERIALS AND METHODS

### A. Sample Collection and Histology

Sheep ovaries were collected from local abattoir and divided into 2 groups. One group was snap-frozen in liquid nitrogen and stored in  $-80^{\circ}\text{C}$  for further usage. The second group was fixed in 10% Neutral Buffered Formalin (NPF) and processed using Tissue-Tek VIP automatic tissue processor and embedded into paraffin wax. Tissue sections of  $5\text{ }\mu\text{m}$  were cut, placed onto super frost slides (HD scientific supplies Pty Ltd, Australia) and stained with Hematoxylin and Eosin for morphological study to identify the different stages of follicular development.

### B. Immunofluorescent Labeling of BMPR1b, FSHR and LHR

Frozen tissues were partially embedded in OCT and  $10\text{ }\mu\text{m}$  sections were prepared using a Cryostat (Carl Zeiss,

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Sydney, NSW, Australia) and fixed in 4% paraformaldehyde at 4 °C for 7 min. Indirect immunofluorescence labeling was performed as previously reported [9]. In brief, 4 µg/ml of the primary antibodies was applied overnight at 4 °C in humidified chamber. The antibodies are (monoclonal) goat anti-BMPR1b (sc-5679), goat anti-FSHR (sc-7798) and goat anti-LHR (sc-26341), all from Santa Cruz Biotechnology, Santa Cruz, CA, USA. After a serial of washing a donkey Anti-goat second antibody conjugated with Alexa 488 (Molecular Probes, Australia) was added for 45 min in dark humidified chamber. Negative controls were performed by omitting the second antibodies for the presence of auto-fluorescent signals and non-specific binding. The sections were mounted using anti-Fade aqueous mounting medium containing 40, 60-diamidino-2-phenylindole (DAPI; Molecular Probes, NSW, Australia).

The sections were examined by Carl Zeiss semi-confocal fluorescent microscope equipped with CarlZeiss Digital Camera (200M Axiovert; Carl Zeiss, Sydney, NSW, Australia) and the images were captured using AsioVision 4.2.8 image analyzer software.

### C. 3D Image Analysis and Immunofluorescent Intensity Quantification

For image acquiring, the exposure time was adjusted using control sections incubated with pre-immune serum in order to subtract auto-fluorescent and non-specific binding background. Surplus fluorescent signals, appeared after such subtraction, were considered specific binding, which were subjected for quantification study. Z-stacks of 10x1 µm optical frames/sections, generated from the 10 µm thick physical sections, were captured and compiled to generate 3D images. The 3D images were used to quantify the intensity of immunofluorescent signals in the entire 10 µm sections (Fig. 1).

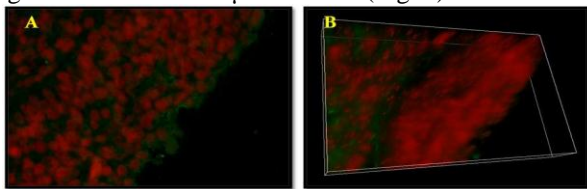


Figure 1. Granulosa cells from pre-ovulatory follicles A. 2D image to determine the Localization and receptor expression B. 3D image for immunofluorescent quantification. X40

### D. Statistical Analysis

Statistical analysis was performed using prism version 6 (Graph Pad Software, La Jolla, CA, USA). The results were analyzed using the mean and standard deviation of BMPR-1b, FSHR and LHR expression in four stages of follicular development and expressed as mean pixel/µm<sup>2</sup> ± SEM.

## III. RESULTS

### A. Morphological Assessment

Four stages of follicular development have been identified and subjected to this study (Fig. 2A-D). Primordial follicles (Fig. 2A) consist of a primary oocyte

surrounded by few spindle-shaped cells, granulosa cells (GCs). The primary follicle (Fig. 2B) initially consists of a primary oocyte surrounded by a complete layer of cuboidal GCs. The zona pellucid (ZP), a thick layer composed of glycoprotein and acid proteoglycans, forms between the oocyte and GCs. Once GCs proliferate and arrange into multiple layers the secondary follicle is formed (Fig. 2C). The Graafian follicle is characterized by a large, fluid-filled antrum and an eccentric oocyte (Fig. 2D).

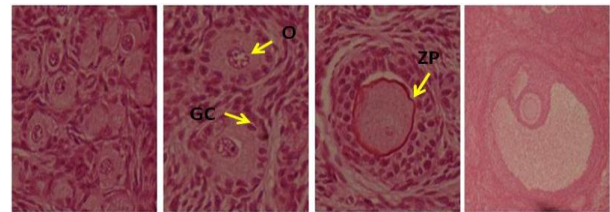


Figure 2. Four stages of follicle development A) Primordial follicle B) Primary follicle C) Secondary follicle D) pre-ovulatory follicle.

### B. Immunofluorescent Localization of BMP1b, FSHR and LHR

Immunofluorescence microscopy revealed that the intensity of a positive immunolabelling of the membrane-bound receptors varies based on the stage of follicular development with the least signals captured in primordial follicles. BMPR1B was expressed in follicular cells of all stages (Fig. 3A-D). Immunolabelling of FSHR showed no staining in follicle cells of primordial follicles but expressed in primary follicles onward (Fig. 3E-H). LHR expression was absent in both primordial (Fig. 3 I) and primary follicles, which then expressed in later stages of follicular development (Fig. 3 I-L).

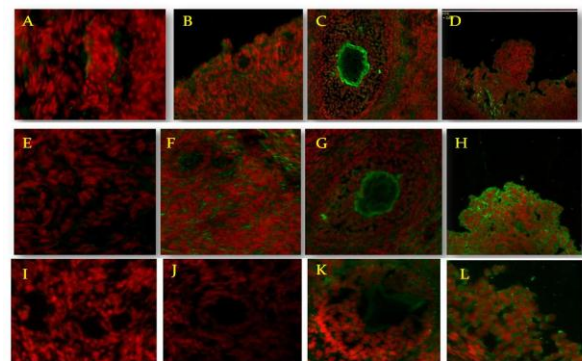


Figure 3. The expression of BMPR1b (A-D), FSHR (E-H) and LHR (I-L). A, E, I, primordial follicles; B, F, J, primary follicles; C, G, K, secondary follicles; D, H, L, Graafian follicles. Green color staining indicates a positive labeling of the receptors. E, I and J show negative staining of FSHR (E) and LHR (I, J). X40

### C. 3D Image Quantification of BMP1B, FSHR, and LHR

Expression during different follicular stages Quantitative analysis based on the intensity of fluorescent signals expressed in 3D images showed that the expression of BMPR1b, FSHR and LHR significantly ( $p < 0.0001$ ) increases with follicular development (Fig. 4A-C). The intensity of labeling was higher for BMPR1b and lower for LHR. BMPR-1b expression was detected in the GCs in all follicular stages of follicular development (Fig.

4A). The FSHR expression was absent in primordial follicles but expressed in the rest stages of follicular development (Fig. 4B). LHR expression was absence in both primordial and primary follicular and expressed in the rest stages of follicular development (Fig. 4C).

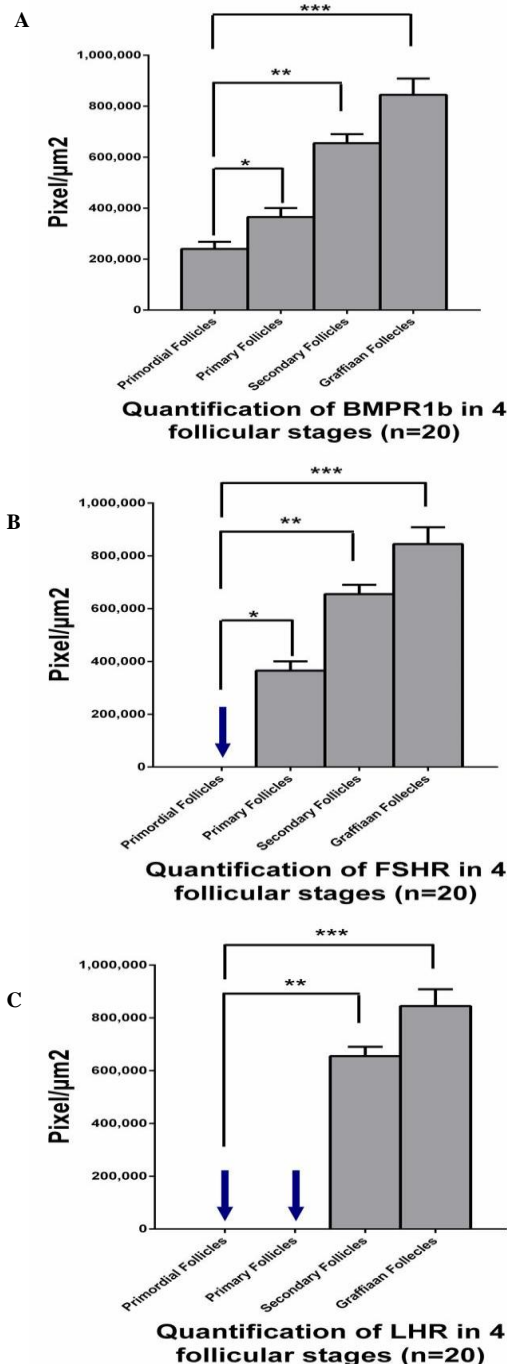


Figure 4. Showed the quantification of receptor expression (pixel/μm<sup>2</sup>) in 4 stages of ovarian development. A) The BMPR1b expression B)FSHR expression C) LHR expression, number of follicles n=20. \*\*: p<0.001, \*\*\*: p<0.0001

#### IV. DISCUSSION

This study demonstrates for the first time the temporo-spatial localization and quantification of BMPR1b, FSHR and LHR in sheep ovaries throughout the initial steps of folliculogenesis. The results indicate that

gonadotropins are not involved in the recruitment of primordial follicles clarifying the ambiguity of the literatures [10], [11]. In addition, the in situ localization of these three receptors in the follicles across several stages of development unequivocally shows the interplay between these hormones and growth factors in the regulation of ovarian function. Traditionally, the detection and quantification of immunofluorescent labeling is conducted by manual subjective methods presented in form of scores range from 0 (no staining) to 4 (intensive staining). This kind of studies may be subjected to bias and therefore non-reliable particularly for the purpose of statistical significance [8]. In addition, subjective approach is not efficient to detect and estimate a small amount of signals, such as within the cell membrane, within a complex structure. Furthermore, semi-quantitative method can only estimate signals emitted from the surface of a single section, which does not represent the actual amount of molecules in the 3D tissue structure. The present study provides alternative approach using computer-based quantitative analysis of 3D objects.

Ovarian folliculogenesis operates mainly under the control of FSH and LH; their levels are regulated by the hypothalamic-pituitary-gonadal axis [2]. However, it has been reported that normal follicle development occurs in mice with mutation in FSH  $\beta$  subunit and FSHR [12], suggesting that the regulation of initial follicle recruitment is gonadotropin-independent [13]. Instead, it is now believed that the initial recruitment of primordial follicles is a gonadotropin-independent process but controlled by intraovarian factors [14] such as the BMPs [15], yet other conflicting reports indicated otherwise [10], [16]

In this study, the absence of FSHR and LHR in primordial follicles unequivocally indicates that the early stage of follicular development is gonadotropin independent. However, the precocious expression of FSHR in primary follicles suggested that FSH may be involved indirectly by up-regulating the expression of BMPR1b [6].

#### V. CONCLUSION

The current study highlights the necessity and feasibility of using non-bias computerized method to detect and quantify the presence and distribution of a small quantity of molecules in a 3D complex structure such as the ovarian follicles. The outcome of the study is important in expanding our understanding of the role of BMPs in the regulation of ovarian functions.

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Dr. Ghanim Almahbobi has gained his PhD in reproductive endocrinology from University of Caen, France in 1987. His postdoctoral training was at the University of New South Wales in Sydney from 1988 to 1993. He worked as a senior scientist at Monash University's Institute of Medical Research for 12 years before he was appointed as a Senior lecturer at Curtin University in 2005. He has obtained two NH&MRC research grants and three other grants from Monash IVF Foundation as a chief investigator and published more than thirty papers and invited reviews in high impact factor journals within the field.



**Sarmed Al-Samerria** Born in Australia 1980, currently is 3rd year doctorate candidate in the school of Biomedical sciences, Curtin University, Australia. His research is under supervision of Dr. Ghanim Almahbobi. His doctorate work to investigate the role of Bone Morphogenetic protein in the regulation of ovarian function in different species using different experimental approaches. He gained a post-grad higher Diploma 2004 in medical technology and BSc (2002) in human biology and microbiology both from Baghdad University. He worked as demonstrator at institute of medical technology, Baghdad. In 2004 he served as scientist in the research centre of the radioactive and biohazard materials, ministry of technology and sciences, Iraq. Sarmed has participated in several national and international conferences and have several publications in the area of reproductive biology.

# The impact of passive immunisation against BMPRII and BMP4 on follicle development and ovulation in mice

S Al-Samerria, I Al-Ali<sup>1</sup>, J R McFarlane<sup>1</sup> and G Almahbobi

School of Biomedical Sciences, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Kent Street, Bentley, Perth, Western Australia 6102, Australia and <sup>1</sup>Centre for Bioactive Discovery in Health and Ageing, University of New England, Armidale, New South Wales 2351, Australia

Correspondence should be addressed to G Almahbobi; Email: g.almahbobi@curtin.edu.au

## Abstract

The primordial follicle reserve is the corner stone of female fertility and determines the longevity and quality of reproduction. Complete depletion of this reserve will lead to primary infertility, and the key-limiting step of follicle depletion is the transition from primordial to primary follicles. It has been reported that this process is gonadotrophin-independent, but other conflicting reports are indicated otherwise and this discrepancy needs to be unequivocally clarified. The aim of this study was to investigate the role of bone morphogenetic proteins (BMPs) in the regulation of folliculogenesis in mice passively immunised against BMP receptor 1B (BMPRII) and BMP4. While a stereological study revealed that the numbers of primordial follicles in immunised mice were significantly higher when compared with control animals, treatment with equine chorionic gonadotrophin showed no effect. In parallel, immunofluorescence microscopy revealed the presence of BMPRII but not FSH receptor in primordial follicles. The number of primary follicles in immunised mice were also significantly increased when compared with control animals. After puberty, the rates of depletion of primordial and primary follicles were increased with age, particularly in treated animals; however, there was no significant difference between the treatment groups of the same age. Based on these results together with our previous reports in sheep and mice, we confirm that the attenuation of BMP signalling system can be an effective approach to sustain the primordial follicle reserve while promoting the development of growing follicles, ovulation and consequently overall female fertility.

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## Introduction

In the ovary, primordial follicles contain arrested primary oocytes, which remain viable throughout life. Among other factors, the numbers and quality of these follicles determine the longevity of female fertility. Every day a few primordial follicles begin development into primary follicles, of these only a small fraction will be destined to complete full folliculogenesis and ovulate, while all the rest die via atresia at some point along the follicular development pathway (McGee & Hsueh 2000). This process of continuous follicular atresia with age and repetition of ovulation after puberty results eventually in complete depletion of this fertility reserve and hence the cessation of ovarian function in mammals and specifically menopause in women. Therefore, the initial transition from primordial to primary follicles, also known as initial follicle recruitment (McGee & Hsueh 2000, Depalo *et al.* 2003, Thomas & Vanderhyden 2006), is the key limiting step of folliculogenesis and thus follicle depletion.

Ovarian folliculogenesis operates mainly under the control of gonadotrophins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), whose levels are regulated by the hypothalamic–pituitary–gonadal axis (Fauser & Van Heusden 1997). Specifically, it has been commonly believed that FSH may be involved in the initial recruitment of primordial follicles (Meredith *et al.* 1992, Flaws *et al.* 1997). However, recent observations have demonstrated that these follicles develop normally in mice lacking active FSH or FSH receptor (FSHR) due to gene mutation in FSH  $\beta$  subunit (Siegel *et al.* 2013) or FSHR (Dierich *et al.* 1998), suggesting that initial follicle recruitment is gonadotrophin independent (Orisaka *et al.* 2009). Instead, this process appears to be regulated by several intraovarian factors (Tanwar *et al.* 2008), such as the bone morphogenetic proteins (BMPs) (Lee *et al.* 2001, Knight & Glister 2003, Abir *et al.* 2008), and this discrepancy requires further investigation.

BMPs are members of the TGF- $\beta$  superfamily and signal through their serine/threonine kinase receptors (Massague 1998), which are of two types: type I

(BMPRIA and BMPRIB) and type II (Cheifetz *et al.* 1987, Chang *et al.* 2002, Derynck & Zhang 2003). The BMP signalling system comprises several BMP ligands, BMPR type I and II, and the intracytoplasmic transcription factors SMADs (Shimasaki *et al.* 2004).

BMP4 mRNA is primarily expressed in theca cells, whereas BMPRIB is predominantly expressed in the granulosa cells of rat ovaries (Shimasaki *et al.* 1999, Erickson & Shimasaki 2003, Tanwar & McFarlane 2011), indicating a paracrine mode of action. BMPRIB is the main and common receptor used by several intraovarian BMPs such as BMP 2, 4, 7, and 15 (Shimasaki *et al.* 2004). On the other hand, the dominant intraovarian BMP4 exerts a major role in the formation of primordial germ cells (oocytes) (Shimasaki *et al.* 1999, 2004), recruitment of primordial to primary follicles in sheep (Ruoss *et al.* 2009) and rat (Nilsson & Skinner 2003), oestradiol production, and inhibition of progesterone secretion and ovulation (Lawson *et al.* 1999, Shimasaki *et al.* 1999, Nilsson & Skinner 2003, Ruoss *et al.* 2009).

In rodent models, the role of BMPs in initial follicle recruitment and overall ovarian function has been investigated, including *in vitro* culture of mouse follicular cells (Shimasaki *et al.* 2004) and cultured whole ovaries of rat (Nilsson & Skinner 2003). We have supported these *in vitro* studies using *in vivo* models namely the Booroola sheep (Ruoss *et al.* 2009) and mice passively immunised against BMP4 (Tanwar *et al.* 2008), both models are characterised by attenuated action of the BMP signalling system, similar to that initially described in *Bmp4*<sup>lacZneo</sup> haplo-insufficient mice (Lawson *et al.* 1999). Our recent report has confirmed that the attenuation of the BMP signalling system by a natural point mutation of BMPRIB in Booroola sheep reduces the rate of primordial follicle recruitment, while increasing primary follicle survival (Ruoss *et al.* 2009). However, it has not yet been determined whether such an effect of BMPs is a sheep species and/or a Booroola strain specific phenomenon or can be applied to other species and different experimental conditions. Although we have confirmed the role of BMP4 in primordial follicle recruitment in mice after passive immunisation (Tanwar *et al.* 2008), this preliminary report was based only on immediate observations after a short-term treatment and using manual counting of follicles.

The aim of this study was to investigate the role of the BMP signalling system in the regulation of primordial follicle depletion in an *in vivo* model of mice passively immunised against BMPRIB and BMP4, using a computer-based stereological method (Braendgaard & Gundersen 1986, Myers *et al.* 2004, Ruoss *et al.* 2009). Specifically, the study aimed to quantify the numbers of primordial and primary follicles and calculate their ratios in different treatment and age groups of mice. The role of gonadotrophins and their interaction with the BMP system in follicular development and ovulation were also examined.

## Materials and methods

### Antibodies

Antibodies to BMP4 (JMCK#54) and BMPRIB (JMCK#59) were raised in chickens against synthetic peptides (Invitrogen Australia) equivalent to amino acids 88–102 (ISMLYL-DEYDKVVLK) of mouse BMP4 and amino acids 103–117 of mouse BMPRIB (NKDLHPTLPPLKDRD). The antibodies were raised and characterised in our laboratory as described previously (Tanwar & McFarlane 2011). In brief, the synthetic peptides were conjugated to diphtheria toxoid (CSL) and emulsified in Freund's complete adjuvant (Sigma-Aldrich Pty Ltd) for the primary vaccination and in Freund's incomplete adjuvant for booster injections. The primary vaccination contained 50 µg of peptide, while all boosters contained 25 µg of peptide. The antibodies were purified from egg yolks using a combination ammonium sulphate and octanoic acid as described by McKinney & Parkinson (1987). The cross reactivity of BMP2 and BMP7 was <0.5% for the anti-BMP4 antibody and the specificity of the BMPRIB antibody was determined by competitive binding ELISA test against random peptides (Tanwar & McFarlane 2011). The DELTA-BLAST analysis showed that the sequence was specific to mouse BMPRIB with no potential binding to other proteins. These antibodies were used to passively vaccinate mice using 50 µg of purified antibody in 100 µl of saline injected subcutaneously. The dose of antibody was similar to that we have used previously (Tanwar *et al.* 2008) and was determined by pilot studies.

### Animals and passive immunisation

Female Swiss mice at 21 days of age were housed at the Physiology Animal House facility, University of New England, NSW, Australia, where all the *in vivo* experiments were carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes, and approved by the University of New England Animal Ethics Committee.

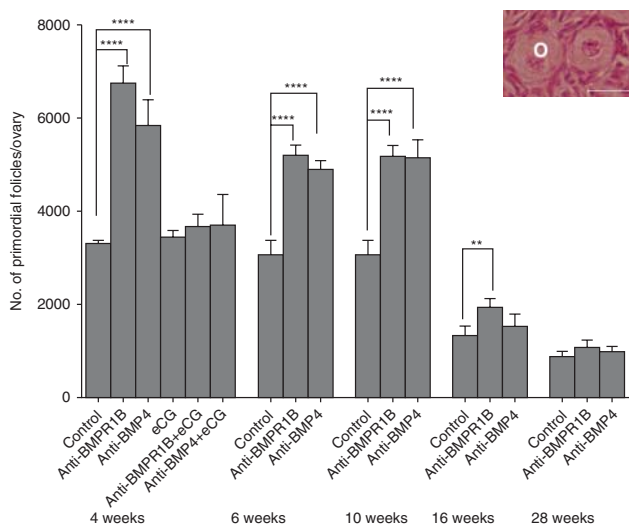
To investigate the effects of attenuating the BMP signalling system on follicle developmental dynamics in the presence and absence of exogenous gonadotrophins, 3-week-old female mice were divided into six groups ( $n=4$  each group) and daily s.c. injections (100 µl) of the following treatments were given for 7 days: the first group, 50 µg anti-BMP4 ( $n=20$ ); the second group, 50 µg anti-BMPRIB ( $n=20$ ); the third group, 1 IU equine chorionic gonadotrophin (eCG; Bioniche Animal Health, Armidale, NSW, Australia) ( $n=4$ ); the fourth group, 1 IU eCG + anti-BMP4 ( $n=4$ ); the fifth group, 1 IU eCG + anti-BMPRIB ( $n=4$ ); and the sixth group, 50 µg purified non-immune chicken IgY, as a control group ( $n=20$ ). Four mice of each group were killed by CO<sub>2</sub> asphyxiation 24 h after the last injection. The remaining animals of first, second, and sixth groups were kept for further periods of 2, 6, 12, or 24 weeks without treatment before they were killed at 6, 10, 16, or 28 weeks of age respectively ( $n=4$  for each group). The total number of animals used per each full experiment was 72 and this experiment was repeated three times (216 mice in total). However, only 72 animals (four animals per treatment group per age), randomly selected from the three experiments, were



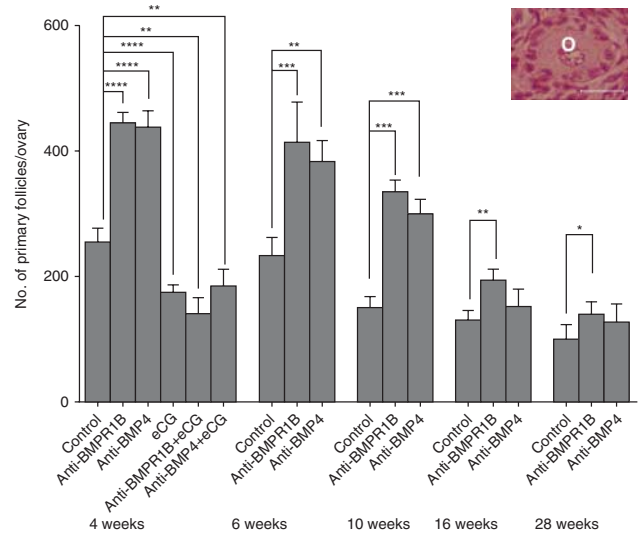
used in this study. All other animals were kept for other investigations. The ovaries were collected and used for further studies as detailed below.

### Ovaries and histology

The collected ovaries were dissected to remove excess surrounding tissues and one from each animal was fixed in Bouin's solution, paraffin embedded, and processed for histological study. The whole ovaries were exhaustively cut into at 5 µm serial sections using a rotary microtome. The sections were consecutively labelled, mounted on a glass slide and stained with haematoxylin and eosin for further stereological studies. For this purpose, the stages of follicle development were classified according to previous reports (Myers *et al.* 2004, Ruoss *et al.* 2009). In brief, primordial follicles appeared as a primary oocyte surrounded by a single layer of squamous granulosa cells (Fig. 1, inset), whereas primary follicles showed a complete single layer of cuboidal granulosa cells (Fig. 2, inset). The same serial sections were used to count the numbers of corpora lutea in each ovary for the determination of relative ovulation rate. Five other ovaries each that represents the five different ages of control animals were used for immunofluorescence studies of receptor expression. These ovaries were snap frozen in liquid nitrogen and stored at -80 °C before they were cryo-sectioned for immunofluorescence localisation of BMPRII and FSHR.



**Figure 1** Stereological quantification of primordial follicles in different treatment groups. The estimated follicle number per ovary was calculated in every 10th section (50 µm interval) using a computerised stereological method. The mean  $\pm$  s.d. of the estimated total number of primordial follicles per ovary was significantly higher in mice passively immunised compared with controls at most ages as indicated. At week 4, eCG treatment either alone or in combination with antibodies showed no difference compared with controls. \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . Inset, a group of primordial follicles showing oocytes (O) enclosed by flattened squamous follicular cells. Bar = 50 µm.



**Figure 2** Stereological quantification of primary follicles in different treatment groups. The estimated follicle number per ovary was calculated in every 10th serial section (50 µm interval) using a computerised stereological method. The mean  $\pm$  s.d. of the estimated total number of primary follicles per ovary was significantly higher in mice passively immunised with anti-BMPRII compared with control group at all animals ages. In mice immunised with anti-BMP4, a significant difference was found up to age 10 weeks. At week 4, eCG treatment with or without antibodies revealed significant reduction in primary follicle numbers. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . Inset, a primary follicle shows an oocyte (O) surrounded by a complete layer of cuboidal granulosa cells. Bar = 50 µm.

### Stereology of ovarian follicles

The estimated total numbers of primordial and primary follicles in the entire ovary were counted by the physical dissector method as originally described (Braendgaard & Gundersen 1986, Gundersen *et al.* 1988). Every 10th section (50 µm interval) selected in the order generated by the microtome was used to provide a non-biased representation for the whole ovary. The section boundaries were determined under low magnification for the placement of the 150  $\times$  150 µm (22 500 µm<sup>2</sup>) sampling grid, and the section sampling was performed as previously described (Myers *et al.* 2004) to generate 32–72 sampling frames. Follicle counting was done using  $\times 40$  oil immersion objective ( $N_A = 1.0$ ), fitted to an Olympus BX microscope. The microscope supported by a computerised Stereo Investigator station (Stereo Investigator software version 8, MicroBrightField, Inc., Colchester, MA, USA) was used for the data collection and analysis. The procedure of follicle counting has been fully described previously (Ruoss *et al.* 2009).

### Estimation of ovulation rates

The estimation of ovulation rates in mice from different treatment groups was carried out by counting the numbers of (new and old) corpora lutea present in each selected ovary, obtained from treated and control animals of 4, 10, and 16 weeks of age ( $n = 3 \times 4 = 12$ ). The numbers of the corpora lutea

**Table 1** Mean estimated total numbers and ratios of primordial follicles in different ages of animals.

Treatment	4 weeks ( <i>n</i> =4 in each group)		6 weeks ( <i>n</i> =4 in each group)		10 weeks ( <i>n</i> =4 in each group)		16 weeks ( <i>n</i> =4 in each group)		28 weeks ( <i>n</i> =4 in each group)	
	Mean $\pm$ s.d.	Ratio	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)
Control	3290 $\pm$ 37	NA	3025 $\pm$ 167	92	2663 $\pm$ 40	81	1329 $\pm$ 99	40	878 $\pm$ 57	27
Anti-BMPRI1B	6741 $\pm$ 185	NA	5183 $\pm$ 116	77	5679 $\pm$ 198	84	1939 $\pm$ 85	29	1070 $\pm$ 75	16
Anti-BMP4	5831 $\pm$ 281	NA	4893 $\pm$ 86	84	4509 $\pm$ 193	77	1517 $\pm$ 137	26	989 $\pm$ 53	17

The ratios of primordial follicles, expressed as a percentage of the estimated total numbers at a given age to the numbers at week 4, gradually decreased with age. There was no significant difference in the ratio of follicle loss between the different groups of treatment at a given age. *n*, number of animals.

were counted in every 40th section of the ovaries, in the order generated by the microtome, leaving 200  $\mu$ m intervals that are slightly larger than the average size of individual corpus luteum in mice ovaries (Sandrock *et al.* 2009). This method provides a non-biased representation of the whole ovary and avoids multiple counting of the same corpus luteum.

### Immunofluorescence localisation of receptors

The ovarian cryosections (10  $\mu$ m) were mounted on Superfrost slides (HD Scientific Supplies Pty Ltd, Australia) and used for indirect immunofluorescence labelling as described previously (Hall & Almahbobi 1997, Al-Samerria & Almahbobi 2014). Briefly, the tissue sections were fixed in 4% paraformaldehyde, and polyclonal goat anti-BMPRI1B (sc-5679) and anti-FSHR (sc-7798) (Santa Cruz Biotechnology) were applied at 4  $\mu$ g/ml overnight at 4 °C. For the negative control, the tissue sections were incubated with 10% (v:v) pre-immune goat serum (Life Technology) instead of primary antibodies. A polyclonal rabbit anti-goat IgG second antibody conjugated to Alexa 488 fluorochrome (A-11078, Life Technology) was applied to all slides at 4  $\mu$ g/ml for 45 min at room temperature. The slides were washed and mounted in Prolonged Diamond anti-fade mounting media with DAPI (P-36962, Life Technology). The slides were examined using a Carl Zeiss semi-confocal microscope equipped with a Carl Zeiss Digital Camera 200 M Axiovert (Carl Zeiss, Sydney, NSW, Australia) and images were captured using AsioVision 4.2.8 image analyzer software (Carl Zeiss).

### Calculation and statistical analyses

Statistical analysis was performed using two-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons to compare the estimated numbers between control and treated animal groups in Prism version 6 (Graph Pad Software, La Jolla,

CA, USA) and the data were presented as  $\pm$  s.d. The differences between means were considered significant when  $P < 0.05$ . The ratios of primordial and primary follicle numbers in different ages of animals were calculated as the percentages of follicle numbers at a given age relevant to the numbers of follicles in animals of the same treatment group at week 4 of age, as given in Tables 1 and 2.

## Results

### Stereological quantification of primordial and primary follicles in different ages

Regardless of treatment, the overall mean of estimated total numbers of primordial and primary follicles per ovary significantly decreased with age, with a noticeably sharp reduction at the later age of animals. Specifically, at the 28th week of age, the follicle numbers were significantly ( $P < 0.0001$ ) reduced in each individual treatment group when compared with those at week 4 (Tables 1, 2 and Figs 1, 2). Only 16–27 and 29–39% of the primordial and primary follicles, respectively, were found in mice at week 28 of age compared with those at week 4. The reduction in follicle amount showed no significant difference neither between the two immunised animal groups nor between the immunised and control animals (Tables 1 and 2).

### Stereological quantification of primordial follicles in different treatment groups

The estimated total numbers of primordial follicles in animals immediately after treatment with anti-BMPRI1B and anti-BMP4 antibodies at 4 weeks of age were significantly ( $P < 0.0001$ ) higher (205 and 177%, respectively) than that in control mice (Fig. 1). The numbers

**Table 2** Mean estimated total numbers and ratios of primary follicles in different ages of animals.

Treatment	4 weeks ( <i>n</i> =4 in each group)		6 weeks ( <i>n</i> =4 in each group)		10 weeks ( <i>n</i> =4 in each group)		16 weeks ( <i>n</i> =4 in each group)		28 weeks ( <i>n</i> =4 in each group)	
	Mean $\pm$ s.d.	Ratio	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)	Mean $\pm$ s.d.	Ratio (%)
Control	254 $\pm$ 12	NA	232 $\pm$ 15	91	149 $\pm$ 9	59	130 $\pm$ 8	51	100 $\pm$ 12	39
Anti-BMPRI1B	444 $\pm$ 8	NA	412 $\pm$ 32	93	334 $\pm$ 9	75	193 $\pm$ 9	43	139 $\pm$ 10	31
Anti-BMP4	438 $\pm$ 13	NA	382 $\pm$ 17	87	300 $\pm$ 11	68	151 $\pm$ 14	34	127 $\pm$ 13	29

The ratios of primary follicles, expressed as a percentage of the estimated total numbers at a given age to the numbers at week 4, gradually decreased with age. There was no significant difference in the ratio of follicle loss between the different groups of treatment at a given age. *n*, number of animals.

remained significantly ( $P < 0.0001$ ) higher at 6 and 10 weeks, but showed a sharp reduction in older animals. Although the numbers were still elevated in both treated groups beyond week 10, a significant difference ( $P < 0.01$ ) was only observed in animals treated with anti-BMPRIB at 16 weeks of age. At 4 weeks, there was no difference in the numbers of primordial follicles between the control and those animals treated with eCG. However, treatment with eCG abolished the immunisation-induced higher numbers of primordial follicles (Fig. 1).

### Stereological quantification of primary follicles in different treatment groups

Analysis of these data showed that the estimated total numbers of primary follicles in animals immediately after treatment with anti-BMPRIB and anti-BMP4 antibodies at 4 weeks were significantly ( $P < 0.0001$ ) higher (175 and 172% respectively) than that in control mice (Fig. 2). In animals treated with anti-BMPRIB, the numbers of primary follicles remained significantly higher at 6 ( $P < 0.001$ ), 10 ( $P < 0.001$ ), 16 ( $P < 0.01$ ) and 28 ( $P < 0.05$ ) weeks. In animals treated with anti-BMP4, the numbers of primary follicles remained significantly higher at 6 ( $P < 0.01$ ) and 10 ( $P < 0.001$ ) weeks, but no significant difference was found at 16 and 28 weeks (Fig. 2).

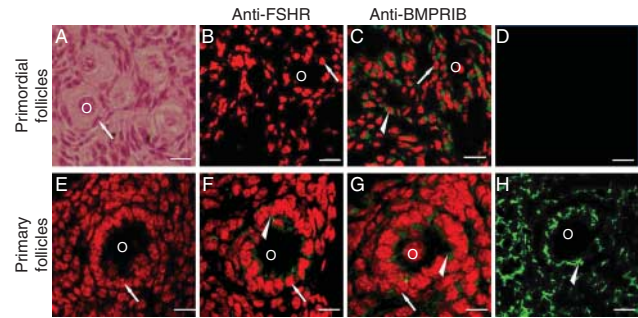
In contrast to the case of primordial follicles, treatment with eCG alone or as a supplement with anti-BMPRIB significantly reduced ( $P < 0.0001$ , 59%) the estimated numbers of primary follicles at 4 weeks. Similar results were observed after treatment with anti-BMP4 ( $P < 0.01$ ) (Fig. 2).

### Immunofluorescence localisation of BMPRIB and FSHR

Immunofluorescence observation revealed positive staining for BMPRIB in both primordial and primary follicles, as well as in the stromal cells (Fig. 3C, G and H). Staining of FSHR showed positive labelling only in the granulosa cells of primary follicles (Fig. 3F), but not in primordial follicles (Fig. 3B). The negative control specimen using pre-immune serum instead of primary antibodies against BMPRIB or FSHR showed no staining in either follicular or stromal cells (Fig. 3D).

### Ovulation rates

A considerable increase in ovulation rate relative to age was observed in all animals irrespective of the types of treatment, particularly in control animals (Fig. 4A). Treatment with eCG revealed a significant increase in ovulation rate at 4 weeks of age when given alone ( $P < 0.0001$ ), with anti-BMPRIB ( $P < 0.001$ ) or with anti-BMP4 ( $P < 0.01$ ) (Fig. 4A). Treatment of animals with



**Figure 3** Immunofluorescence localisation of BMPR-IB and FSHR. (A) A histological section stained with H&E, showing a group of primordial follicles. (B and C) Groups of primordial follicles showing negative staining for FSHR (B) and positive staining for BMPRIB in follicular cells (C, green). (D) A negative control section using pre-immune serum showing no receptor staining. (E, F, G and H) Primary follicles reveal positive staining (green) for both FSHR (F) and BMPRIB (G) in granulosa cells. (E) A primary follicle, as shown in (F), observed with a single filter for DAPI, revealing nuclear staining (red) only. (H) A primary follicle, as shown in (G), observed with a single filter for Alexa 488, revealing only positive receptor staining (green) in granulosa and stroma cells. Arrows indicate follicular/granulosa cells stained with DAPI (red). Arrowheads point to positive signals of antibody binding (green). O, oocyte. Bar = 50 µm.

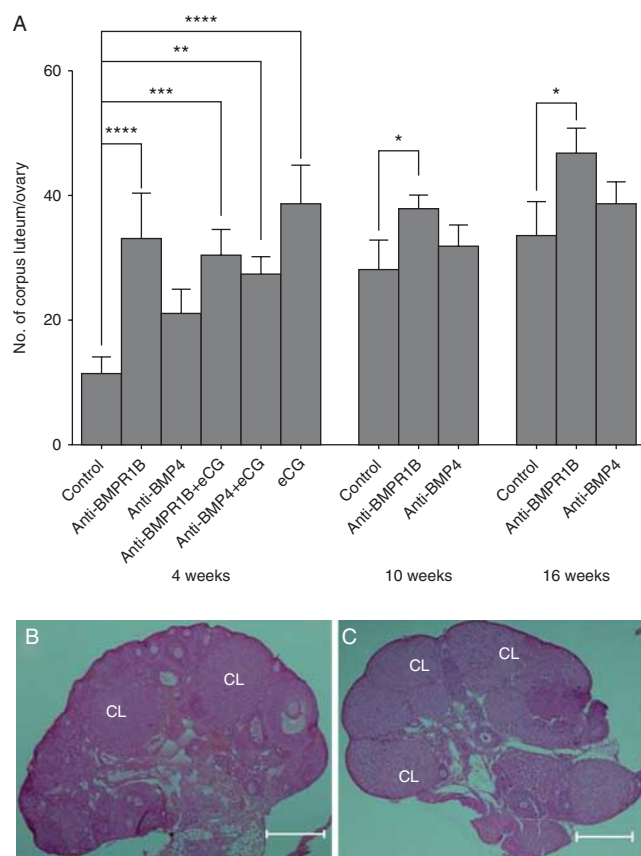
anti-BMPRIB resulted in a significant increase in ovulation rates at 4 ( $P < 0.0001$ ), 10 and 16 ( $P < 0.05$ ) weeks when compared with control animals (Fig. 4A, B and C). However, although treatment with anti-BMP4 resulted in elevation in ovulation rate across the ages, this was not significant (Fig. 4A).

### Discussion

This study describes how short-term attenuation of the BMP signalling system (1 week) by passive immunisation against BMPRIB and BMP4 can sustain the reserve of primordial follicles while increasing the numbers of developing primary follicles and the rate of ovulation in pre-pubertal mice *in vivo*. Together with our previous reports (Tanwar *et al.* 2008, Ruoss *et al.* 2009), we extend our knowledge regarding the role of the BMP signalling system in the regulation of follicular development. This includes the stimulatory role of these factors in the initial recruitment of primordial follicles to the pool of growing follicles in the rat (Nilsson & Skinner 2003) and sheep (Ruoss *et al.* 2009), also their stimulatory role in the ovulation rate and formation of primordial germ cells and oocytes as previously documented (Moore *et al.* 2004, Lawson *et al.* 1999, respectively).

In this study, the resulting changes induced by passive immunisation against the BMP signalling system on both the initial recruitment of follicles and ovulation rate remained for several weeks after treatment. Considering the short half-life of IgG antibody, such an extended effect for several weeks after immunisation was unlikely to have resulted from the direct action of the antibodies.





**Figure 4** Ovulation rates in animals of different treatment groups. (A) The ovulation rates were estimated in three groups of mice by manually counting the corpora lutea in every 40th serial section (200 µm interval) of individual ovaries. In addition to the increase in ovulation rates with age, the mean  $\pm$  s.d. of ovulation rates were significantly higher in treated animals with anti-BMPRIIB or eCG with or without antibodies. (B and C) Histological sections showing more corpora lutea in the ovary of treated animals with anti-BMPRIIB (C) than in control animals (B). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . CL, corpus luteum. Bar = 100 µm.

Instead, we believe that the immediate significant increase in primordial follicle population in treated animals compared with controls would have taken several weeks before it was abolished and returned to the normal condition. It is also possible that the noticeably higher magnitude of age-related acceleration of ovulation rate in control animals compared with the treated ones would have over-ridden the significant difference in ovulation rates between the two groups of animals at later ages. These results are similar to those observed in Booroola sheep (Ruoss *et al.* 2009). However, the receptor mutation-induced effect in sheep is permanent, resulting in an increased longevity of reproduction (Ruoss *et al.* 2009). In our passive immunised mouse model, the effect is transitory so when the 'effector' is removed the system returns to normal. Nonetheless, the exact intracellular pathway that mediates the effect of passive immunisation on BMP signalling system needs to be clarified.

Interestingly, the effect of immunisation on the preservation of primordial follicles and increasing ovulation rates using anti-BMPRIIB was stronger than when using anti-BMP4 antibody. This may be explained by the fact that BMPRIIB is a common receptor used by several other BMPs in addition to BMP4 (Nilsson & Skinner 2003, Shimasaki *et al.* 2004). Nonetheless, the significant effect induced by BMP4 immunisation (>80% of effect induced by anti-BMPRIIB) indicates that BMP4 is likely to be the most effective BMP among other ligands in the process of primordial follicle formation (Lawson *et al.* 1999) and recruitment (Tanwar *et al.* 2008) in mice and perhaps less so in later stages of follicular development.

After puberty and irrespective of treatment, the significantly increased degree of depletion of primordial and primary follicles with age is expected, possibly due to increased post-pubertal follicular development and late cyclic recruitment of mature follicles (Broekmans *et al.* 2007). In support of this, the rate of ovulation was increased with age, therefore indicating that the maturity of animals increased reproductive activity.

Based on our observations, it is interesting to mention that the numbers of primordial follicles in treated and untreated animals continue to decrease with age of mice (these results) and sheep (Ruoss *et al.* 2009). However, the pattern of such reduction appears species-specific. While in sheep a dramatic decline was observed during the early stages of life (from birth to 1.5 year, Ruoss *et al.* 2009), it is only observed in older mice at the age of 16 and 28 weeks. In contrast, the numbers of primordial follicles in untreated mice remain unchanged up to the age of 10 weeks. The sustained numbers of primordial follicles over this relatively long period of mouse life may support the previous reports demonstrating the existence of postnatal renewal of primordial follicles in this species (Johnson *et al.* 2004, Kerr *et al.* 2006) and more recently in human (White *et al.* 2012).

More interestingly, in treated animals the numbers of primordial follicles are doubled after immunisation. Such a significant increase is unlikely due to the attenuation of primordial follicle depletion alone. Instead, it appears that in the early-postnatal period of mice, the role of BMPs is not only limited to the stimulation of primordial follicle recruitment but also inhibition of the formation of new primordial germ cells and primordial follicles, a process which reportedly exists in this species (Johnson *et al.* 2004, Kerr *et al.* 2006). However, a stimulatory action of BMPs on this process has been reported during mouse foetal development (Lawson *et al.* 1999). Nonetheless, the numbers of primordial follicles in both treated and untreated animals declined to the level similar to that in older animals of different species and postmenopausal women.

While eCG treatment has remarkable effects on the rate of ovulation as expected, the failure of eCG to induce a detectable effect on initial recruitment of

primordial follicles supports the notion that this process is gonadotrophin-independent (Lee *et al.* 2001, Orisaka *et al.* 2009, Al-Samerria & Almahbobi 2014) and that there is not a direct action of gonadotrophins on primordial follicles. In support of this, we have found that while the expression of BMPRII was detectable in follicular cells of mouse primordial follicles, FSHR was absent but later appeared in primary follicles. These data together with a previous report in rat (Moore & Shimasaki 2005) contradict previous reports (Meredith *et al.* 1992, Flaws *et al.* 1997) and therefore suggest the presence of a complex interplay between gonadotrophins and other growth factors such as BMPs where direct and indirect cross-signalling regulates ovarian function (Tanwar & McFarlane 2011). The reported decrease in primordial follicles after eCG treatment (Tanwar *et al.* 2008) conflicts with the present result probably due to the manual counting method used in the previous study instead of the automated stereology used in this work. The fact that injection of eCG together with antibody either unexpectedly abolished the effect of immunisation on primordial and primary follicles or reduced the immunisation-induced increases in ovulation rates, requiring further investigation. Meanwhile, we suggest that eCG may act on the larger follicles whereby its action indirectly antagonises the action of the antibodies on the small follicles. Together with previous studies, this study extends our further knowledge regarding the important role of BMPs but not gonadotrophins in the recruitment of initial primordial follicle.

Despite the reduction in the rates of transition of primordial follicles to primary follicles (loss/depletion) in treated animals, the estimated total numbers and ratios of primary follicles derived from primordial follicles in these animals after passive immunisation were significantly increased rather than decreased. This may suggest a reduction in apoptosis, allowing more primary follicles to survive, which requires further investigation. Also, as BMPs exert a proliferative action on granulosa cells, promoting early folliculogenesis and oestrogen production (Guangqin *et al.* 2013) including the transformation of primary follicles to further developmental stages, immunisation against BMPRII will slow this action hence resulting the accumulation of more primary follicles.

The novelty of these results may appear inconsistent with a previous study in rat such as that reported by Nilsson & Skinner (2003). In rat, the addition of more BMPs to cultured ovaries stimulated primordial follicle recruitment, simply monitored by a increased number of primary follicles therefore decrease number of primordial follicles (Nilsson & Skinner 2003). However, in this study, the attenuation of BMP action by immunisation inhibits primordial follicle recruitment monitored by preservation of these follicles in ovaries when compared with untreated animals. We believe that the significant increase in primordial follicle number in treated animals

may also be due to other factors such as stimulation of *de novo* formation of primordial follicles. On other hand, the increase rather than decrease in the numbers of primary follicles possibly due to inhibition of apoptosis and the transition of primary follicles to developing antral follicles.

In this regard, the previously observed reduced proportion of primary follicles to the total number of the whole follicle population in the ovaries of anti-BMP4-treated mice (Tanwar *et al.* 2008) was probably due to the significant increase in the numbers of primordial follicles after immunisation. As in the case of primordial follicles, the increase in numbers of primary follicles reduced gradually with age after reaching its peak at 10 weeks of age.

In contrast to the case of primordial follicles, eCG treatment alone and in combination with either treatment significantly reduced the numbers and ratios of primary follicles. Evidently, this effect reported in this study cannot be due to a reduced recruitment of primordial to primary follicles, because eCG has no effect on the numbers of primordial follicles and expression of FSHR was not found in primordial follicles. Instead, it is likely that the reduced numbers and ratios of primary follicles resulted from the eCG-induced acceleration of further development of primary follicles and transformation to the stages of secondary and preantral follicles, processes that are promoted by gonadotrophins (Broekmans *et al.* 2007, Orisaka *et al.* 2009). Clearly, the more the age of animal the more accelerated the follicle development occurs, and less persistent primary follicles were found as previously reported in older ewes (Ruoss *et al.* 2009). This effect was more obvious in treated animals probably because of reduced recruitment rates of primordial to primary follicles.

The significant increase in the ovulation rate in mice treated with anti-BMPRII mimics the well-characterised feature of Booroola sheep (Mulsant *et al.* 2001, Souza *et al.* 2002, Ruoss *et al.* 2009), which exhibit mutation in BMPRII (Wilson *et al.* 2001, Campbell *et al.* 2006, Ruoss *et al.* 2009). It should be noted that the estimated total numbers of counted corpora lutea represent accumulated ovulation of multiple cycles at a given age. No attempt was made to exclude corpora lutea that were formed and persisted before the treatment because this is a comparative study between treated and untreated animals within each individual age group. The numbers of corpora lutea formed from cycles before the time of treatment were equally present and accounted for in all treated and untreated animals.

The underlying mechanism of BMP action in the regulation of ovulation is not well understood (McNatty *et al.* 2007). However, it has been reported that BMPs inhibit the gonadotrophin-induced progesterone production by maturing follicles (Xia *et al.* 2003, Pierre *et al.* 2004, McNatty *et al.* 2007), in addition to its inhibitory action on final cell differentiation (Cejalvo *et al.* 2007),

in this case granulosa cell luteinisation. In another study, we have found that protein expression of BMPRII in granulosa cells obtained from IVF patients is significantly downregulated in dominant follicles (Regan *et al.* 2012). It appears that the downregulation of BMP signalling system is a pre-requisite for the late follicle maturation, granulosa cell differentiation and progesterone formation hence ovulation.

In conclusion, this study demonstrates that the role of the BMP signalling system is likely to be an important regulator of ovarian function, particularly the processes of follicle development, depletion and ovulation. These results, together with our previous studies in mice (Tanwar *et al.* 2008), sheep (Ruoss *et al.* 2009) and humans (Regan *et al.* 2012) and other studies in mice and rats (Lawson *et al.* 1999, Erickson & Shimasaki 2003, Nilsson & Skinner 2003), confirm that this role of BMPs in the regulation of ovarian function is applicable across different species and experimental conditions. We propose that the attenuation of BMP signalling system is an effective approach to increase female fertility reserve while promoting follicle development, ovulation and eventually the overall fertility.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## Chapter 5 : Protein expression of BMPR-1B, FSHR and LHR in granulosa cells of mice after passive immunisation against BMPR-1B and Bmp-4

S. Al-Samerria<sup>1</sup>, J. McFarlane<sup>2</sup> and G. Almahbobi<sup>1\*</sup>

<sup>1</sup> School of Biomedical Sciences, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Perth, WA, 6102, Australia.

<sup>2</sup> Centre for Bioactive Discovery in Health and Ageing, University of New England, Armidale, NSW 2351, Australia.

Short title: BMP Receptor 1B regulate follicle development

Key words: BMPs, receptors, immunisation, gene expression, immunofluorescence BMP-4, Bone Morphogenetic Protein 4; BMPR-1B, Bone Morphogenetic Protein Receptor 1B; FSHR, Follicle Stimulating Hormone Receptor; LHR, Luteinizing Hormone Receptor; eCG, Equine chorionic gonadotropin.

\*To whom correspondence should be addressed:

Ghanim Almahbobi, PhD  
School of Biomedical Sciences, Curtin University  
Kent St, Bentley, WA, 6102, Australia.  
Telephone: +618 9266 1192  
Email: g.almahbobi@curtin.edu.au



## 5.1 Abstract

The transition of primordial follicles to primary follicles is a crucial step in controlling female oocyte reserve and length of reproductive life. Gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH) are well known to regulate follicle development by enhancing the granulosa and theca cells proliferation and steroid production. Recently, several reports have indicated that bone morphogenetic proteins (BMPs) play important regulatory roles in recruitment of follicles, ovulation rate and initial stages of follicle development, which was found to be gonadotropin-independent. The aim of the current study is to investigate the mechanism of interaction, between the gonadotropin receptor expression and the BMP signalling during follicle development in mice passively immunised against BMP receptor 1B (BMPR-1B) and BMP-4.

The outcomes of the current study showed tightly coordinated mechanisms of action between BMP signalling and gonadotropin receptor expression. First, it shows that the role of BMPs in follicle development extends beyond the early stages of follicle development, confirmed by significantly increased expression of its receptor (BMPR-1B) in mice treated with anti-BMPR-1B. This effect of BMPR-1B attenuation was associated with increased expression of the gonadotropin receptors (FSHR and LHR). This regulatory interaction between BMPs and gonadotropin was found to be independent of BMP-4 (another important member of BMPs involved in transition from primordial follicle to primary follicle). Gonadotropin hormones were appeared to have no role in early stages of follicle development (primordial follicle stage). The role of equine chorionic gonadotropin eCG in regulating and enhancing the gonadotropin action mediated by BMPR-1B was also revealed from this study. The outcomes of the current study suggested that the BMP signalling system is not only involved in regulating the early stages of follicle development but also involved indirectly in regulating the later stages of follicle development by increasing gonadotropin hormones receptor expression, which lead to increases the follicular somatic cell sensitivity toward gonadotropin hormones. Attenuation of BMPR-1B could be exploited in further studies as an effective tool to attain better or enhanced reproductive life in female.

## 5.2 Introduction

Follicle development is the key process of female productivity and it is responsible of producing single mature oocyte ready for fertilization. This process consists of series of chronological and well-regulated steps starting from primordial follicle formation and ending in production of single mature follicle ready for fertilization (McGee and Hsueh, 2000). Very few primordial follicles from the recruited population reach ovulation, while the vast majority die of atresia. In humans, the quality of these follicles determines the length of reproductive life and fertility. The follicle development process was thought to be mainly regulated by the actions of gonadotropin hormones mainly FSH and LH. However, recent studies including mutated version of FSH receptor in mice were able to demonstrate that the initial stages of follicle development to be FSH independent (Siegel et al., 2013), and found to be regulated by intra-ovarian factors (Al-Samerria et al., 2015), such as the BMP (Lee et al., 2001b, Glister et al., 2004, Abir et al., 2008). The BMP signalling pathway was reported to be involved directly in the initial stage of follicle formation (Lawson et al., 1999, Nilsson and Skinner, 2003, Knight and Glister, 2006), primordial follicle recruitment (Lee et al., 2001a, Nilsson and Skinner, 2003, Sun et al., 2010), ovarian steroidogenesis (Shimasaki et al., 1999, Hosoya et al., 2014), follicle cyclic recruitment (McGee and Hsueh, 2000), ovulation (Moore et al., 2004, Al-Samerria et al., 2015), and follicle atresia (Shimasaki et al., 1999, Araújo et al., 2010). Hence, a coordinated inter-network between the gonadotropin hormones and BMP signalling systems is appeared to be existed. Several recent studies have revealed clues to these interactions. For instance, BMP-6 was shown to increase the expression of FSH receptors (FSHR) in human granulosa cells *in vitro* (Shi et al., 2010), on the other hand an inhibitory action of BMP-2, BMP-4 and BMP-15 on FSHR formation was reported in granulosa cells in chicken (Haugen and Johnson, 2010), ewes (Faure et al. 2005) and rats (Otsuka et al., 2001), respectively. However, detailed interactions between BMP signalling and gonadotropin hormones in the regulation of follicle development are yet to be determined. BMP receptors are categorized as of two types (type I and type II), which activate the BMP signalling through ligand binding and activating of a cytoplasmic transcription factors. BMPR-1B is the common receptor used by several intra-ovarian BMPs such as BMP 2, 4, 7 and 15 (Shimasaki et al., 2004). BMP-4, a major intra-ovarian BMP, is involved in formation of PGCs (oocytes) (Shimasaki et al., 2004, Shimasaki et al., 1999), recruitment of primordial to primary follicles

in *Booroola* sheep (characterised by natural point mutation in the BMPR-1B) (Ruoss et al., 2009) and rat (Nilsson and Skinner, 2003), estrogen production and inhibition of progesterone secretion and ovulation (Lawson et al., 1999, Shimasaki et al., 1999, Nilsson and Skinner, 2003, Ruoss et al., 2009). However, whether the interactions of BMPR-1B and other BMP ligands are gonadotropin dependent is not known. Therefore, the present study investigates the interaction between BMP signalling (BMPR-1B and BMP-4) and gonadotropin hormones using immunofluorescence detection technique and computer-based analysis to quantify the receptors expression intensity in 3D images, specifically the localisation of the membrane bound receptors of the BMPs, FSH and LH in primary, secondary and Graafian follicle stages of mice ovaries. To this end, an *in vivo* mouse model with attenuated BMP signalling was generated using passive immunisation against BMPR-1B and BMP-4 ligand according to (Tanwar et al., 2008, Tanwar and McFarlane, 2011, Al-Samerria et al., 2015) with an aim to demonstrate the reciprocal regulation at their receptor expression level during ovarian follicle development.

## 5.3 Materials and methods

### 5.3.1 Animal care and passive immunisation

Female Swiss mice were kept at the Animal House facility, University of New England, Armidale, NSW, Australia. All *in vivo* experimental procedures were carried out with according to the Australian code of practice and were approved by the University of New England animal ethics committee. Attenuation of BMP signalling was achieved by passive immunisation with anti-BMP-4, anti-BMP-1B and the role of gonadotropin hormones in regulating follicle development were demonstrated by injecting the animal with exogenous eCG (Bioniche Animal Health, Armidale, NSW, Australia) as previously described in (Tanwar and McFarlane, 2011, Al-Samerria et al., 2015). Briefly, 3-weeks old female mice were divided into 6 groups (N=4); each group received 100 µl of subcutaneous injections of the following treatment. This experimental procedure was repeated thrice and a total of 72 animals were used for this study. Four mice from each group were killed by CO<sub>2</sub> asphyxiation, 24 h after the last injection and at the end of the 4th week. The animals were immediately processed to obtain the ovaries. One ovary from each animal was snap-frozen in liquid LN<sub>2</sub> and stored at -80 °C for 2 weeks before being cryosectioned for *in situ* immunofluorescent localisation and quantification of immunofluorescent intensities of the receptors.

**Table 5-1: Animal treatment groups**

Group	Treatment
Group 1	n=4, 50 µg of purified non-immune chicken IgY
Group II	n=4, 50 µg of anti-BMP-1B antibody
Group III	n=4, 50 µg of Anti-BMP-4 antibody
Group IV	n=4, 1 IU of eCG
Group V	n=4, 1 IU of Anti-BMP-1B+ eCG
Group VI	n=4, 1 IU Anti-BMP-4+ eCG

### 5.3.2 Antibodies

The antibodies for BMP-4 and BMP-1B used in the passive immunisation experiments were raised and fully characterized including their cross reactivity and sequences in our laboratory as previously described (Tanwar and McFarlane, 2011, Tanwar et al., 2008). The synthetic peptides equivalent to the amino acid 88-102 (ISMLYLDEYDKVVLK) for mouse BMP-4 and aa

103-117 for mouse BMPR-1B (NKDLHPTLPPLKDRD) (Invitrogen Australia, Mount Waverly, Vic, Australia). The antibodies were raised in chickens and purified from egg yolks. Purification of antibodies was done using ammonium sulphate and octanoic acid in a combination according to McKinney and Parkinson (1987). Binding specificity and absences of cross reactivity of anti-BMP-4/anti-BMPR-1B antibodies were reconfirmed for this study using ELISA based test against random peptides and DELTA-BLAST analysis respectively.

### **5.3.3 Immunofluorescent labelling of BMPR-1B, FSH and LH receptors**

The ovaries were snap-frozen in liquid LN<sub>2</sub> before being used for *in situ* immunofluorescent localisation and quantification of BMPR-1B, FSH and LH receptors. Whole frozen ovaries were partially embedded in OCT and cut into 10 µm sections using a cryostat (Carl Zeiss, Sydney, NSW, Australia). Tissue sections were mounted on to Superfrost slides (HD scientific supplies Pty Ltd, Australia) for immunofluorescent procedures as described in (Al-Samerria and Almahbobi, 2014). Briefly, fixation was done using 4% paraformaldehyde at 4°C for 7 min. Antibodies staining were done using polyclonal anti-BMPR-1B (sc-5679), anti-FSHR (sc-7798) and anti-LHR (sc-26341) raised in goat at 4°C for overnight in humidified chamber. These antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The negative control was prepared by replacing the first antibody with 4 µg/ml of purified goat IgG (02-6102) (Life Technologies, Mulgrave, Victoria, Australia). After a serial washing, a rabbit anti-goat antibody conjugated with Alexa-488 fluorochrome (Life Technologies, Mulgrave, Victoria, Australia) used in 4 µg/ml concentration for 45 min at room temperature in dark and humidified chamber. All sections were mounted using an anti-fade aqueous mounting medium containing 40, 60-diamidino-2-phenylindole (DAPI; Molecular Probes, NSW, Australia). Sections were analysed using inverted confocal microscopy (AR1+/A1+; Nikon Corporation, Tokyo, Japan). Images were captured using A1+ software (Nikon Corporation, Tokyo, Japan).

### **5.3.4 Image analysis and Quantification of Immunofluorescent Intensities of the receptors**

Quantification of immunofluorescent intensities of the receptors were done in the granulosa cells of the Graafian follicles by placing three ( $9 \times 10^3$ ) µm<sup>3</sup> sections in three different locations of the follicle and the mean of intensities at the regions of interest (ROI) were used in the calculation (Figure 2A). The same principle was applied for secondary follicles with a ( $1 \times 10^3$ ) µm<sup>3</sup> sections (Figure 2B) and primary follicle with 250 µm<sup>3</sup> in two locations of the follicles

(Figure 2C). The comparison performed between different treatments of the follicles in the same stages and not between follicles across various stages. Images were captured and calculation of intensities of fluorescent signals in different stages of follicle development was performed as described by Al-Samerria and Almahbobi, (2014). In brief, the exposure time was adjusted using rabbit non-immune serum instead of the 1st antibody, the fluorescent single generated was taken as the base to subtract the signals generated by auto-fluorescence and non-specific binding. A surplus of fluorescent signal generated after subtraction was considered as a positive signal and used in the quantification analyses. For generation of 3D images, 10  $\mu\text{m}$  thick physical sections were scanned and Z-stacks of  $10 \times 1 \mu\text{m}$  optical frames/sections were generated. These Z-stacks were compiled in order to generate the 3D images, which were used for quantification of intensity of the immunofluorescent signals from the whole 10  $\mu\text{m}$  tissue section using Volocity 3D image analysis software, version 6.2 (Perkin Elmer; Waltham, MA, USA).

### **5.3.5 Statistical analysis**

Immunofluorescent expression intensities at the three different stages of follicle development (primary, secondary and Graafian follicles) between the treated and untreated animal were analysed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. The data are presented using Prism version 6 (Graph Pad Software, La Jolla, CA, USA) as means  $\pm$  standard deviations. The differences were considered significant at  $P < 0.05$ .

## **5.4 Results**

### **5.4.1 Immunofluorescent localisation of receptors in ovarian follicle**

In untreated control animals, there was no fluorescence signal observed in the negative control samples incubated with non-immune serum instead of the 1st antibody (Figure 1A, B). The positive immuno-labelling localisation of BMPR-1B, FSHR and LHR was demonstrated in all stages of follicle development including Graafian follicles (Figure 1).

While FSHR was observed only in granulosa cells (Figure 1D, H), the expression of BMPR-1B and LHR was observed in both granulosa and theca cells in all samples regardless of the treatment (Figures 1C, 1E, 1F and 1G). After treatment with anti-BMPR-1B antibody, stronger signals of BMPR1B (Figure 1G) or FSHR (Figure 1H) were observed.

#### ***5.4.2 In situ quantification of BMPR-1B, FSHR, and LHR in three different stages of follicle development***

Quantitative analysis, based on the intensity of the fluorescent signals from the representative 3D images, of BMPR-1B, FSHR, and LHR expression was conducted in animals passively immunised against BMPR-1B, BMP-4 with or without eCG in three stages of follicle development (Graafian follicle, Figure 3; secondary follicle, Figure 4 and primary follicles, Figure 5). All these analyses were carried out on the signals collected from granulosa cells only. The selection of the follicle development stages were done according to Pedersen & Peters' (1971) classification scheme (Pedersen and Peters, 1971). In Graafian follicles, the expression of BMPR-1B, FSHR and LHR showed significant increases after treatment with anti-BMPR-1B antibody with or without eCG (Figure 3A-C). Treatment with eCG alone also caused significant increases in the expression of BMPR-1B and FSHR (Figure 3A, B, respectively) but not in LHR (Figure 3C). In contrast, immunisation with BMP-4 antibody alone or with eCG administration did not show any significant change in the expression of either receptor, when compared to the level of expression in control animals (Figures 3A-C). In secondary follicles, expression of BMPR-1B, FSHR and LHR was significantly increased after treatment with anti-BMPR-1B alone (Figure 4A-C). Treatment with anti-BMPR-1B plus eCG induced significant increase in the expression of BMPR-1B only but not FSHR or LHR. None of other treatments caused any significant change in either receptor. In primary follicles, only the treatment with anti-BMPR-1B with or without eCG induced significant increase in the expression of BMPR-1B only (Figure 5A-C). Regardless of the treatments, the LHR expression was completely lacking in the primary follicles (Figure 5C).

## 5.5 Discussion

The present study was designed to investigate the mechanism of interaction between the gonadotropin hormones and the BMP signalling during follicle development in mice. The study was implemented by using passive immunisation of selective BMPR and ligands. The localisation of the receptors was visualized by *in situ* immunofluorescent staining followed by computer-based quantitative analysis. It should be kept in mind that this kind of immunofluorescence technique is not efficient enough to detect the small changes of immunofluorescent signals. Moreover, they represent only the signals that are reflected from the surface of sections and thus do not represent the amount of molecules in the tissue structure. However, using the 3D image quantification analysis enabled us to measure the fluorescent signals generated by immunofluorescent labelling and correlated with the receptor protein expression, hence, the expression level of the receptors can be interpreted indirectly in terms of the intensities. This method has already provided a promising result (Al-Samerria and Almahbobi, 2014), hence used in the present study.

The two gonadotropic hormones, (FSH) and (LH), believed to play primary roles in controlling ovarian function and in particular the early stage of follicle development (Flaws et al., 1997). However, we have demonstrated a positive expression of BMPR-1B and lack of expression of FSHR in the follicular somatic cells of primordial follicles in sheep (Al-Samerria and Almahbobi, 2014) and mice (Al-Samerria et al., 2015). This observation suggested that early stage of follicle development was mainly regulated by the action of BMP signalling not the gonadotropins. These observations supported by other reports suggested that initial stages of follicle recruitment being gonadotropin-independent (Jaatinen et al., 1996). Furthermore, the involvement of intra-ovarian factors, such as BMPs, in this process has directed us to investigate the actual coordinated mechanism involved between the gonadotropin hormones and the BMP signalling (Tanwar et al., 2008, Ruoss et al., 2009).

In the present study, the changes in the protein expression of the BMPR-1B, FSHR and LHR in the follicular somatic cells (granulosa and theca cells) induced by passive immunisation in mice against BMPR-1B and BMP-4 demonstrated the tightly coordinated mechanisms of action between BMP signalling and the gonadotropin hormones. The increased ovulation was shown to be due to BMPR-1B natural attenuation in *Booroola* sheep and passively immunised mice (Ruoss et al., 2009, Al-Samerria et al., 2015). Even, indirect up-regulation of BMPR-1B by



FSHR was demonstrated previously (Al-Samerria and Almahbobi, 2014). Extending to these observations, the present study showed that later stages of follicle development (in secondary follicles and Graafian follicles) are also regulated by BMPR-1B indirectly by increasing the receptors' expression (increase the number of the receptors) and ultimately leading to increased sensitivity of the follicular somatic cells toward gonadotropin hormones. Similar increased in the follicular somatic cell sensitivity towards gonadotropin has also been linked with enhanced cellular proliferation (Findlay and Drummond, 1999), reduced apoptotic activity (Zhang et al., 2013), luteinisation and increased steroidogenic activity (androgen, oestrogen and progesterone production) (Miyoshi et al., 2006, Wang and Roy, 2009). The significant increase of the BMPR-1B expression in mice vaccinated against BMPR-1B with/without eCG could be due to the observed up-regulation of FSHR and LHR, which was supported by similar pattern of up-regulation in the expression of FSHR and LHR which caused due to point mutation in BMPR-1B in *Booroola* sheep (Regan et al., 2015). The localisation of BMPR-1B and LHR in both the granulosa and theca cells compared to the exclusive localisation of FSHR in the granulosa cells in the Graafian follicle of mice vaccinated against BMPR-1B indicated wider spectrum of action of BMPR-1B and LHR than that of FSHR. Further studies can dissect the reason of specific localisations of these receptors with regard to their function in later stages of follicle development.

The up-regulation of BMPR-1B, FSHR and LHR due to attenuation of BMPR-1B was demonstrated in the secondary follicles similar to that of Graafian follicles. The up-regulation of BMPR-1B expression could be due to the auto-compensation effect to overcome the inhibitory action of the vaccination. Up-regulation of BMPR-1B expression in granulosa cells of secondary follicles indicated that BMPs role in follicle development is also extended beyond the early stages of the follicle development. It was also supported by the observations of BMP signalling being essential for granulosa cells including cell proliferation and differentiation (Shimasaki et al., 2004) as well as for the oocyte-granulosa cells communication (Otsuka et al., 2001, Yi et al., 2001). This observation implied that follicle development process is tightly controlled by the BMPs action directly by regulation at the early stages for follicle development and indirectly by enhancing the granulosa cells sensitivity by up-regulation of gonadotropin action (FSHR and LHR) leading to enhanced steroidogenic activity (oestrogen and progesterone productions) (Nilsson and Skinner, 2003, Shimasaki et al., 2004, Khalaf et al., 2013, Hosoya et al., 2014). Selective up-regulation of BMPR-1B and FSHR in the Graafian

follicles treated with eCG indicated that eCG could stimulate FSH production, which was also supported by the observation of production of FSH ligand by eCG (Murphy, 2012). FSH ligand could up-regulate FSHR (Findlay and Drummond, 1999), and thus, eCG could indirectly up-regulate BMPR-1B expression in the present study. This observation showed the role of eCG in regulating and enhancing the gonadotropin action mediated by BMPR-1B.

Previously we have demonstrated that attenuation of BMP-4 using passive immunisation increases the total number of primordial and primary follicles in mice (Al-Samerria et al., 2015). This observation indicated that the passive immunisation was effective. However, the attenuation of BMP-4 with/without eCG showed no effects on BMPR-1B, FSHR and LHR protein expression. This observation suggested that the interaction between BMPs and gonadotropin is independent of BMP-4, possibly occurring at an intracellular level. It is important to note that the outcome of the current study does not contradict the known fact of BMP-4 being essential for follicle development as reported earlier (Nilsson and Skinner, 2003), but rather restricts its role to be not effective in regulating BMPR-1B, FSHR and LHR expression. BMP-4 was shown to play an important role during the transition from primordial follicles to primary follicles (Tanwar et al., 2008, Al-Samerria et al., 2015), however, whether its involvement is regulated by SMAD signalling or cAMP pathway requires further studies. The unaffected or little reduced expression of the FSHR in all the 3 follicle stages in BMP-4 attenuated mice could be explained by the fact that BMP-4 is known to inhibit epidermal growth factor (Chen et al., 2013), which was again known to down regulate FSHR expression (Findlay and Drummond, 1999). Hence passive immunisation with anti-BMP-4 could thus reduce FSHR expression indirectly through up-regulation of epidermal growth factor (EGF). The attenuation of BMP-4 showed no effects on BMPR-1B, FSHR and LHR expressions in secondary follicle. This observation suggested that the interaction between gonadotropin hormones and BMPs occurred at the level of receptors (intracellular level) not at the level of the ligands (extracellular), however further investigation is required to understand this.

Attenuation of BMPR-1B in the primary follicles resulted in up-regulation of BMPR-1B and had no effects on the FSHR expression. This observation suggested that BMPR-1B expression was pre-requisite for the onset of the follicle development in early stages of follicle development. It could include the stages of PGCs formation, and the transition of the primordial to primary follicles as BMPR-1B was shown to be expressed in granulosa cells of the primordial follicles and no FSHR or LHR expression were detected in sheep (Al-Samerria and Almahbobi, 2014)

and mice (Al-Sammerria et al., 2015). No significant effect on the FSHR and lack of LHR expression in the primary follicles in the present study reconfirmed that gonadotropins (FSHR and LHR) have no direct effects at the early stages of follicle development (Campbell et al., 2009). It was supported by the findings of Campbell et al (2009) that the increase in prolificacy in *Booroola* sheep (with natural point mutation in the BMPR-1B) was not because of the increase in FSH level, but was due to increase in granulosa cells' sensitivity mediated by other intra-ovarian factors such as BMPs. In the present study, up-regulation of BMPR-1B, FSHR and LHR expression in the secondary and Graafian follicles with anti-BMPR-1B with and without eCG could be explained by an auto compensation phenomenon to overcome the inhibitory action of the vaccination. This type of unequivocal interaction between BMPR-1B and the gonadotropin hormones *in vivo* for follicle development has not be shown so far, and can open new roads to study and enhance the reproductive life in female mice and ultimately in humans.

## **5.6 Acknowledgement**

We would like to acknowledge the provision of research facilities and the scientific and technical assistance of the staff of CHIRI Biosciences Research Precinct core facility, Curtin University.

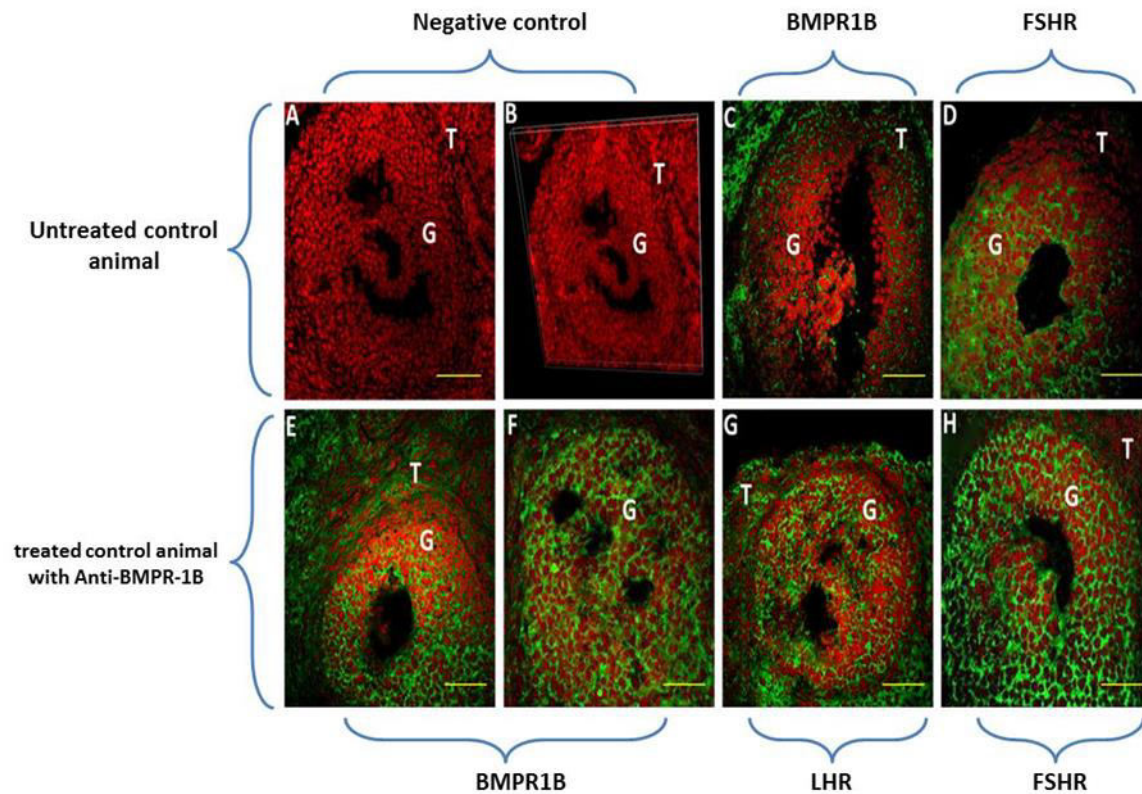
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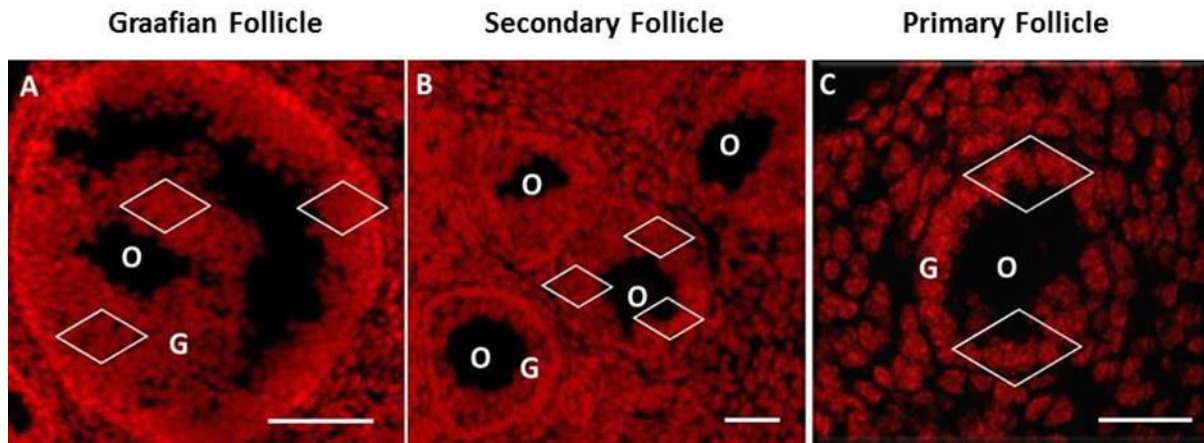
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## 5.8 Figures & Figure Legends



**Figure 5-1: The expression of BMPR-1B, FSHR and LHR using immunofluorescence localisation in Graafian follicles.**

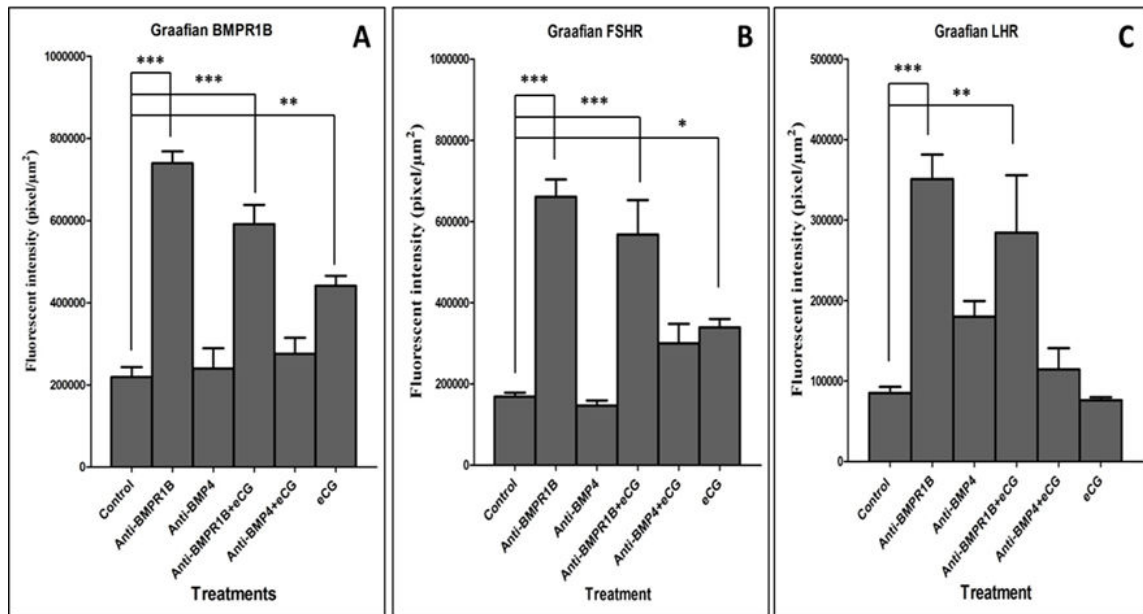
A: negative control with DAPI staining (red staining). B: negative control of 10  $\mu$ m 3D Z-stack. C: BMPR-1B in granulosa and theca cells (green staining). D: FSHR in granulosa cells only. E: BMPR-1B in theca and granulosa cells in animals immunised with anti-BMPR-1B. F: BMPR-1B in theca and granulosa cells in animals immunised with anti-BMPR-1B plus eCG. G: LHR in theca and granulosa cells in animals immunised with anti-BMPR-1B. H: FSHR in granulosa cells in animals immunised with anti-BMPR-1B. G: Granulosa cells, T: Theca cells. Bar=50  $\mu$ m.



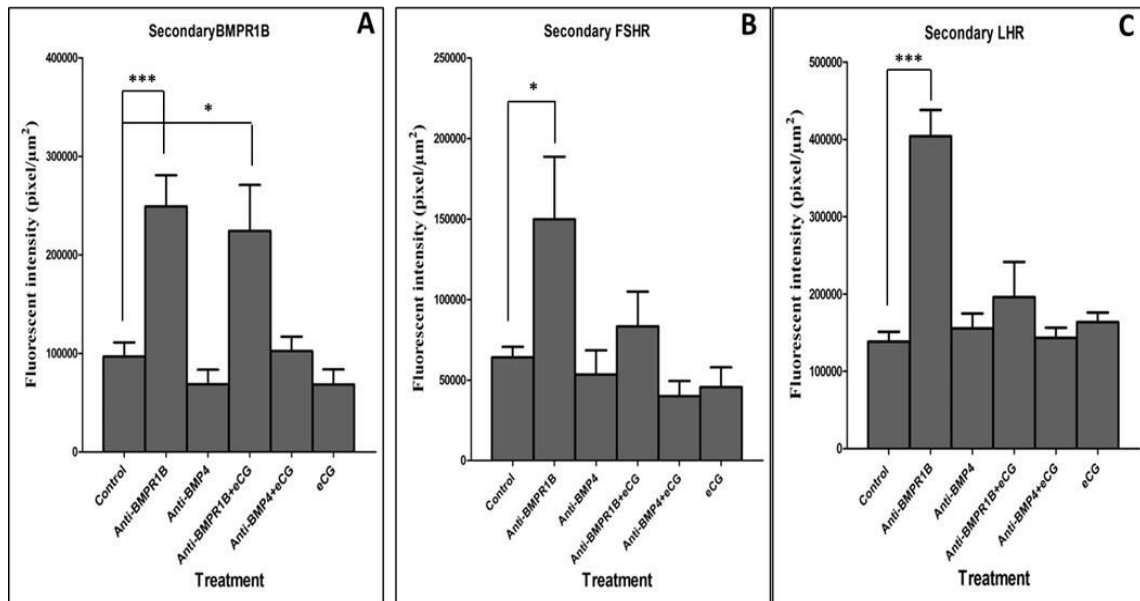
**Figure 5-2: Immunofluorescence quantification.**

A: Graafian follicle, a cubic have been placed in three different locations on granulosa cells ( $10\text{ }\mu\text{m}$  height x  $30\text{ }\mu\text{m}$  width x  $30\text{ }\mu\text{m}$  length)  $\mu\text{m}^3$ , Scale Bar= $50\text{ }\mu\text{m}$ . B: Secondary Follicle, a cubic have been placed in three different locations on granulosa cells ( $10\text{ }\mu\text{m}$  height x  $10\text{ }\mu\text{m}$  width x  $10\text{ }\mu\text{m}$  length)  $\mu\text{m}^3$ , Scale Bar= $10\text{ }\mu\text{m}$ . C: Primary Follicle, a Cubic have been placed in two different locations on granulosa cells ( $10\text{ }\mu\text{m}$  height x  $5\text{ }\mu\text{m}$  width x  $5\text{ }\mu\text{m}$  length)  $\mu\text{m}^3$ , Scale Bar= $5\text{ }\mu\text{m}$ . G: Granulosa cells, O: oocyte.

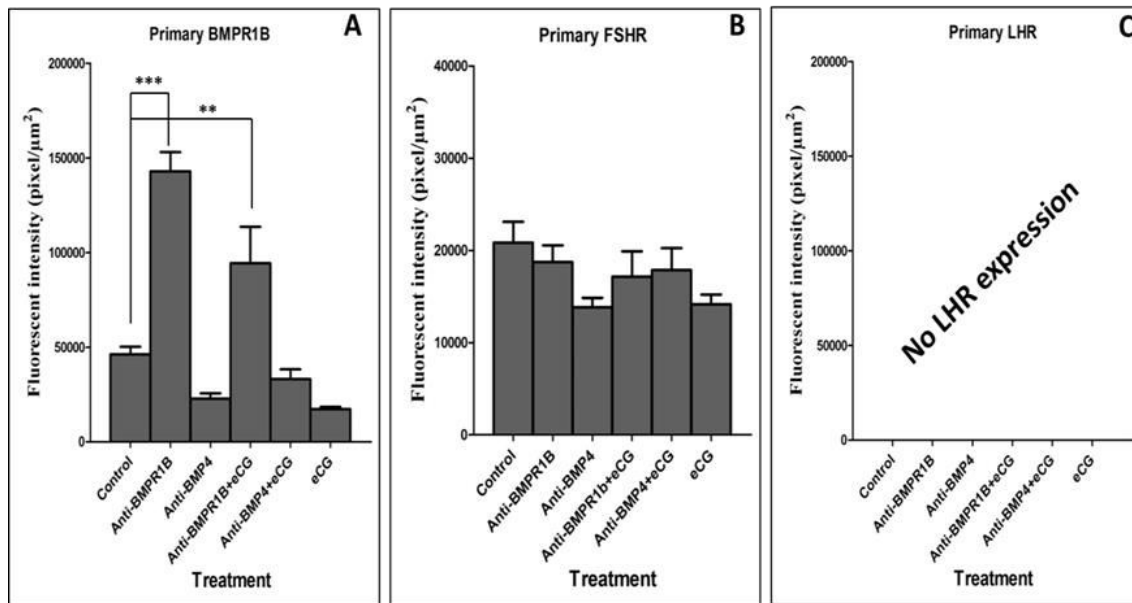




**Figure 5-3: *In situ* quantification of the expression of receptors (pixel/ $\mu\text{m}^2$ ) in Graafian follicles after different treatments. Number of follicles N=20/ovary [\*= 0.05, \* =  $p < 0.001$ , \*\*\* =  $p < 0.0001$ ]**



**Figure 5-4: *In situ* quantification of the expression of receptors (pixel/ $\mu\text{m}^2$ ) in Secondary follicles after different treatments. Number of follicles N=20 per ovary. [ $*$  = 0.05,  $*$  =  $p < 0.001$ ,  $***$  =  $p < 0.0001$ ]**



**Figure 5-5: *In situ* quantification of the expression of receptors (pixel/ $\mu\text{m}^2$ ) in Primary follicles after different treatments. Number of follicles N=20 per ovary. [ $\ast$ = 0.05,  $\ast$ =  $p<0.001$ ,  $\ast\ast\ast$ =  $p<0.0001$ ]**

## Chapter 6 : The Impact of impaired function of BMPR-1B on mRNA expression of FSHR and LHR in mice.

S. Al-Samerria<sup>1</sup>, S. K. A. Palanisamy<sup>2</sup>, I Ali<sup>2</sup>, J. McFarlane<sup>2</sup> and G. Almahbobi<sup>1\*</sup>

<sup>1</sup> School of Biomedical Sciences, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Perth, WA, 6102, Australia.

<sup>2</sup> Centres for Bioactive Discovery in Health and Ageing, University of New England, Armidale, NSW 2351,

Short title: BMPs and gonadotropin interaction

Key words: BMPs, receptors, immunisation, gene expression, immunofluorescence

\*To whom correspondence should be addressed:

Ghanim Almahbobi, PhD  
School of Biomedical Sciences, Curtin University  
Kent St, Bentley, WA, 6102, Australia.  
Telephone: +618 9266 1192  
Email: [g.almahbobi@curtin.edu.au](mailto:g.almahbobi@curtin.edu.au)

## 6.1 Abstract

The bone morphogenetic proteins (BMP) were recently shown to have various important roles in the initial stages of folliculogenesis, which include recruitment of primordial follicles, transition of primordial follicles to primary follicles and increase ovulation rate. Although gonadotropins including follicle stimulating hormone (FSH) and luteinizing hormone (LH) are known to regulate folliculogenesis, recent studies have shown that the early stages of folliculogenesis to be gonadotropin-independent. We have recently demonstrated that modulation of BMP signalling system influences ovarian FSHR and LHR expression. This observation suggested the existence of the interaction between BMP signalling and gonadotropin at the cellular receptor level. The aim of the present study is to investigate if the interaction between BMP receptors signalling systems and gonadotropin (FSHR and LHR) expression is evident at the nuclear or transcript level. The results of this study showed a significant increase in mRNA of both FSHR and LHR in mice treated with anti-BMPR-1B, and thus the effect at the transcript level were confirmed. The involvement of gonadotropin hormones for the induction of FSHR and LHR expression for overall follicle development by administration of equine chorionic gonadotropin eCG at the transcript level was also confirmed. Further, the results confirmed that passive immunisation of BMP signalling can be used to enhance the reproductive life in mice.

## 6.2 Introduction

The BMPs are highly conserved members of the transforming growth factor- $\beta$  superfamily. Among their diverse morphogenetic roles, they are well known for their critical roles in multiple aspects of embryogenesis (Hogan, 1996). Mammalian reproduction is controlled by coordinated and episodic release of gonadotropin releasing hormones and subsequent secretion of the pituitary gonadotropin. Gonadotropins namely the folliclestimulating hormone (FSH) and the luteinizing hormone (LH) are involved in oocyte development, cyclic follicles recruitment and development which is considered as central to female reproduction. Female folliculogenesis progresses through systematically regulated and coordinated processes involving primordial follicle development and recruitment, follicle development, maturation and, finally, production of single mature oocyte required for fertilization (Knight & Glistler 2006). This process of production of mature oocyte is tightly regulated and influenced by several intra-ovarian factors and hormones, since it starts from a population of primordial follicles and ends up with only one mature oocyte. Further, the folliculogenesis process is also responsible for the length of the reproductive life, fertility and the menopause in women. Among the other intra-ovarian factors, the BMP system is known to play a key role in the regulation of gonadotropin regulated folliculogenesis as well as mitosis in granulosa cells (Otsuka, et al., 2011). Recent studies conducted in our laboratory and others have demonstrated the role of BMPs in several stages of folliculogenesis such as ovarian steroidogenesis (Shimasaki et al., 1999, Hosoya et al., 2014), cyclic follicle recruitment (McGee and Hsueh, 2000), formation of the primordial germ cells (Lawson et al., 1999, Nilsson and Skinner, 2003, Knight and Glistler, 2006), recruitment of primordial follicles (Lee et al., 2001a, Nilsson and Skinner, 2003, Sun et al., 2010), regulating ovulation rate (Moore et al., 2004, Al-Samerria et al., 2015) and follicle atresia (Shimasaki et al., 1999, Araújo et al., 2010). Several BMP members, such as BMP-6, BMP-7, and BMP-15 have recently been shown to be directly involved in regulation of gonadotropin function such as prevention of immature ovulation (McNatty, et al 2009), FSH receptor signalling (Huang, et al 2001), FSH synthesis and secretion (Otsuka et al 2002) and initial follicle recruitment (Ruoss et al 2009). For instance, BMP-6 is suggested to be involved in regulating the increase of FSH receptor expression (FSHR) in human granulosa cells *in vitro* (Shi et al., 2010). Further, FSHR synthesis in the pituitary gland was shown to be inhibited by the action of BMP-2, BMP-4 and BMP-15

in chicken granulosa cells *in vitro* (Haugen and Johnson, 2010) and ewe (Faure, et al., 2005) and rat granulosa cells (Otsuka et al., 2001). On the other hand, BMP-4 was shown to control follicle longevity and primordial follicle development (Nilsson and Skinner, 2003). Recent studies conducted in our laboratory have found that the initial stages of follicle recruitment are gonadotropins independent and rather BMPs dependent (Lee, et al. 2001, Knight & Glistler, 2003, Abir, et al. 2008). All these recent studies have led to the suggestion that BMP signalling regulate different stages of folliculogenesis by controlling gonadotropin sensitivity and secretion from pituitary gonadotropins (Takeda, et al 2011).

BMP signalling is transduced through a heterodimeric complex consisting of type I and type II receptors (BMPRI-1 and BMPRI-2), which are transmembrane serine/threonine kinase receptors (Yamashita et al 1996). The BMP receptors transduce the BMP functions by binding with various ligands and activating various transcription factors. BMPRI-1B was shown as the common receptor of several other BMPs such as BMP 2, 4, 7 and 15 (Shimasaki et al., 2004). Therefore, it has been selected to conduct this study. To this end, the present study was designed to analyze the expression level of FSH and LH receptors at transcript level, *in vivo*, in mice ovarian follicle cells, in response to BMP system attenuation (BMPRI-1B and BMP-4) and gonadotropin administration. BMPs attenuation was gained through applying passive immunisation against BMP receptor and ligand, and gonadotropin stimulation by eCG administration as described in our previous studies. The expression was investigated at the transcript level by using quantitative real time qPCR. The study aims to understand the reciprocal interaction between BMP signalling and gonadotropin hormones.

## 6.3 Materials and methods

### 6.3.1 Passive immunisation and experimental animals' growth condition

For passive immunisation, 16 female mice were grown in controlled conditions in the Physiology Animal House facility, University of New England, Armidale, NSW, Australia for the entire experimental period (till four weeks). The *in vivo* experimental procedures conducted in this study were approved by the University of New England Animal Ethics Committee. The experiments adhered to an Australian code of practice. Passive immunisation against (BMPR-1B and BMP-4) was done to attenuate BMP signalling system. And eCG was administered to the animals for gonadotropin stimulation. For this purpose, anti-BMP-4, anti-BMPR-1B and eCG (eCG; Bioniche Animal Health, Armidale, NSW, Australia) were used according to Tanwar and McFarlane (2011) and Al-Samerria et al. (2015). Three-week old female mice were divided into four groups of four each (N=4) and were given 100 µl of antibodies subcutaneously for 7 days as outlined in further details in **Table 1**.

**Table 6-1: Animal groups and treatment**

Group	Treatment
Group 1	n=4, 50 µg of purified non-immune chicken IgY (Control)
Group II	n=4, 50 µg of anti-BMPR-1B antibody
Group III	n=4, 50 µg of anti-BMP-4 antibody
Group IV	n=4, 1 IU of eCG

Mice were killed by CO<sub>2</sub> asphyxiation after 24 h of the last injection and immediately processed for recovery of the ovarian samples. This experimental procedure was repeated thrice and a total of 48 animals were used for this study. Sample ovaries were preserved in RNA Later (Ambion, Austin, TX, USA) at 4 °C for RNA extraction.



### **6.3.2 Antibody preparation**

Antibodies used in this study for passive immunisation were raised against BMP-4 and BMPR-1B in chicken, purified from egg yolks and injected subcutaneously as 50 µg in 100 µl saline as described in (Tanwar and McFarlane, 2011). The doses of the antibodies were determined in the pilot studies. Briefly, the synthetic peptides equivalent to amino acids 88-102 (ISMLYLDEYDKVVLK) for mouse BMP-4 and amino acids 103-117 for mouse BMPR-1B (NKDLHPTLPPLKDRD) (Invitrogen Australia, Mount Waverly, Vic, Australia) were used. These antibodies were generated and fully characterized previously in our laboratory (Tanwar and McFarlane, 2011, Tanwar et al., 2008) for their specificity and cross-reactivity using ELISA test and DELTA-BLAST analysis. Antibody purification was accomplished using ammonium sulphate and octanoic acid in a combination (McKinney and Parkinson 1987). The synthetic peptides were conjugated to diphtheria toxoid (CSL) and emulsified in Freund's complete adjuvant (Sigma-Aldrich Pvt Ltd) for primary vaccination and with Freund's incomplete adjuvant for booster injections.

### **6.3.3 RNA extraction and RT-PCR**

The RNA extraction and reverse transcription of RNA were carried out by following the methodology adapted from Ciller et al., 2016. Briefly, fresh mouse ovaries were collected and stored in RNA Later ® solution (Ambion, Austin, TX, USA) at 4 °C overnight according to the manufacturer's instructions. Left-side ovaries were used for total RNA extraction using TRI reagent as per manufacturer's instructions (T9424: Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). RNA purity was determined using Nano-Drop 8000 (Thermo Fisher Scientific, Inc, Wilmington - USA) and RNA integrity was checked using 1% agarose gel electrophoresis (Le Bourhis et al., 2005). Isolated total RNA was stored at - 80°C until use. 2 µg of total RNA was reverse transcribed to cDNA using Oligo (dT) 15 Primer and M-MLV Reverse Transcriptase (Promega, Alexandria NSW) and in the presence of rRNasin® RNase Inhibitor (Promega, Alexandria NSW) according to Ciller et al., 2016.

### 6.3.4 Primers and qPCR

The reference Beta actin ( $\beta$ -actin) gene primers sequences were as described in Ciller et al., 2016 and the rest of primers used in this study (FSHR, LHR, SDHA and 18s) were designed in-house using NCBI-Primer blast software (Ye et al., 2012) and were synthesized in Gene Works Australia (Thebarton, South Australia SA, 5031, Australia). The primers used and their sequences are presented in Table 2. The specificity of the primers was confirmed by following the methodology described by Ciller et al., 2016. The polymerase chain reactions (PCR) were carried out using a BioRad CFX384 Touch<sup>TM</sup> Real-Time PCR Detection System using Fast EvaGreen qPCR Mix (Biotium, Hayward CA). For qPCR reaction, a 16-80 ng of control ovary cDNA was used. The qPCR was carried out using 2X Fast Plus Green qPCR mix, 16 ng of cDNA, primers of 1 mM final concentration in triplicates using  $\beta$ -actin, SDHA and 18s as reference genes in total volume of 1  $\mu$ l.

**Table 6-2: Primers used in this study**

Target	Forward Primer	Reverse Primer
FSHR	GGAGCCTCTGGGCCAGTCGT	GGAGCCTCTGGGCCAGTCGT
LHR	GGACGACGCTAATCTCGCTGGAG	GGGTAGGTCAGCGTGGCAACC
B Act (Reference gene 1)	CGT CGA CAA CGG CTC CGG CATG	TGG GCC TCG TCA CCC ACA TAG
SDHA (Reference gene 2)	CTT GAA TGA GGC TGA CTG TG	ATC ACA TAA GCT GGT CCT GT
18s (Reference gene3 )	CGG ACA GGA TTG ACA GAT TG	CAA ATC GCT CCACCA ACT AA

### 6.3.5 Statistical analysis

The total number of mice was 16 for one set of experiment (N=16). For quantifying the relative changes in the gene expression in the samples were estimated using the  $2^{-\Delta C_T}$  method adapted from Livak et al (2001). Relative PCR amplification of the target genes were calculated after normalization with the geometric mean of the three reference genes as described by Vandesompele et al., 2002 (Vandesompele et al., 2002). Differences in the expression level between the treated and untreated animal, in whole ovaries were analysed using one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls multiple range test and using the SAS computer software package (SAS Institute Inc., Cary, NC, USA). The differences were considered significant at  $P < 0.05$ .

## 6.4 Results

### ***6.4.1 Relative expression of transcripts of FSHR and LHR after different treatments***

Quantification analysis based on relative levels of transcript expression was carried out to investigate the FSHR and LHR expressions in the whole ovaries of mice immunised against anti-BMPR-1B, anti-BMP-4 and administrated with eCG for gonadotropin stimulation. All results for the whole ovaries. RT-PCR quantification showed similar profiles (Figure 6.1) of gene expression for FSHR and LHR in ovarian homogenates with a significant increase in FSHR and LHR expression in mice treated with anti-BMPR-1B or administrated with eCG, when compared against untreated control animals. Treatment with anti-BMP-4 showed no significant changes of FSHR and LHR mRNA when compared to the untreated control animals. Treatment with eCG showed the highest increase in the FSHR and LHR mRNA expression levels (Figure 6.1).

## 6.5 Discussion

The present study was designed to identify whether the interaction between the gonadotropin and the BMPs occurs at the transcript level. The study was accomplished by investigating the gonadotropins receptor expression (mRNA) in mice passively immunised against BMPR-1B and BMP-4 respectively to attain transient attenuation of the BMP signalling system. The interaction was analysed at the transcript level by comparing the relative expression of mRNA levels after reverse transcription and quantification of cDNA by Real-time PCR. Basically the ovarian cells immunised with BMPR-1B, BMP-4 or administrated with eCG were compared for transcripts of FSHR and LHR in mice ovaries. Although whole ovaries were taken for experimental analysis, the results most properly represent changes in the granulosa and the theca cells. Since FSHR expression and LHR are restricted in these two tissue cells. Hence the granulosa and the theca cells were indirectly targeted for the interaction study by studying expression of FSHR and LHR. The two gonadotropins FSH and LH are the central players in female reproductive function including most of the ovarian function and ovarian folliculogenesis. However, recently it was found that FSH mutant version of mice could retain folliculogenesis (Siegel et al. 2013). Further, positive expression of BMPR-1B and lack of expression of FSHR in the primordial follicles in sheep and mice (Al-Samerria and Almahbobi, 2014; Al-Samerria et al., 2015) confirmed that the early stage of follicle development was

primarily regulated by BMP signalling system. Enhanced expression of the FSHR and LHR due to a point mutation in BMPR-1B (Reagan, et al., 2015) and the observations of indirect up-regulations of BMPR-1B by FSHR (Al-Samerria and Almahbobi, 2014) in the previous studies indicated a definite interaction of BMPR-1B and the gonadotropin receptors which is reflected in the protein level expression of the receptors. In addition to BMPR-1B, BMP-4 is also included in the study since BMP-4 is a dominant intra-ovarian BMP involved primary follicle transition, recruitment and formation of primordial germ cells (Shimasaki et al., 2004, Shimasaki et al., 1999); (Ruoss et al., 2009). Immunisation specific for BMPR-1B resulted in significant increase in the FSHR and LHR transcript levels, which was not observed in case of immunisation with anti-BMP4. This observation was also supported by the weak protein level expression of gonadotropin receptors when using anti-BMP4 compared to anti-BMPR-1B (Al-Samerria et al 2015). The experiments were designed in whole ovaries, thus includes an overall analysis during all stages, irrespective of any specific stage of follicle development. The administration of eCG also resulted in significant increase in the FSHR and LHR transcript levels indicating that the role of gonadotropin in overall follicle development being significant at transcript level. However, early stages of folliculogenesis were previously found to be gonadotropin-independent, albeit at protein level (Orisaka et al. 2009). Further studies can be conducted to ascertain the involvement of FSHR and LHR at transcript levels in the early stages of follicle development in animals administrated with eCG, which could reveal whether the non-dependence of early stages of folliculogenesis from gonadotropin is at transcript level. Thus, the present study extended the knowledge of interaction of BMP receptors with the gonadotropin receptors to transcript level. The interaction appears to occur at the nuclear level, thus the transcription of the gonadotropin receptors is influenced by the absence of the BMP receptors. This level of interaction is novel, and it would be interesting to investigate the factors and regulators of this interaction between the BMP receptors and the gonadotropin receptors. In addition, it would be useful to confirm the changes in BMP signalling by examining the anticipated downstream effects in the signalling pathway including examination of phosphor-Smad 1/5/8 in follicles.

In conclusion the current study reconfirms that attenuation of BMP signalling through passive immunisation can be effectively used to attain and enhance reproductive life in mice.

## 6.6 Acknowledgements

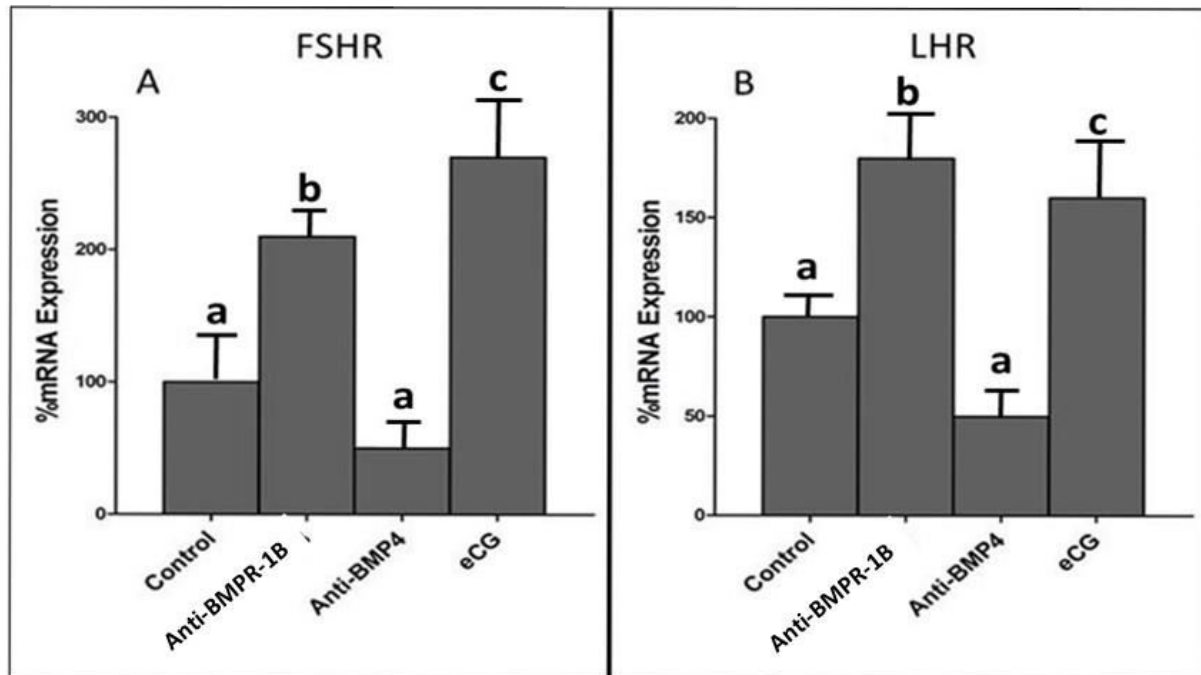
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## 6.8 Figure and figure legends



**Figure 6-1: Relative gene expression (mRNA) levels of FSH and LH receptors in whole ovaries after different treatments. The difference was considered significant at ( $P < 0.05$ )**

A: The FSHR mRNA ( $\Delta C_T$  value) level obtained by qPCR; B: The LHR mRNA ( $\Delta C_T$  value) level obtained by qPCR. B: The LHR mRNA ( $\Delta C_T$  value) level obtained by qPCR (N=4 ovaries and the experiment repeated 3 times).

## Chapter 7 : General discussion

This chapter gives an overview of the four studies undertaken to understand the role of BMP signalling in follicle development, primordial follicle consumption, primordial to primary follicle transition, primary follicle survival, ovulation rate, their receptor expression patterns at the protein and transcript level and also their interaction with the gonadotropins. The complex nature of ovarian structure and the process of folliculogenesis through different stages were a main focus of the study. Previously, the role of BMP signalling in follicle development was studied either through the natural (i.e., *in vivo*) mutation in BMP receptor (Ruoss et al., 2009), in gene knockout model (Zhang and Bradley, 1996), in an *in vitro* method, accessed at molecular or biochemical level (Wang and Roy, 2009) or in an *in situ* method assessed manually through subjective methods of microscopy (Erickson and Shimasaki, 2003). The first aim of my studies was to determine the temporal-spatial expression of the BMP receptors and the gonadotropin hormone receptors (FSHR and LHR) in sheep, during the various stages of follicle development *in situ* by using the non-biased computerized 3D-image analysis system. The results of this study showed for the first time the utility of immunofluorescence labelling combined with computer based intensity quantification using 3D-image analysis, compared to previous methods. The approach enabled more precise quantification and gave statistically significant findings which would not have been possible with the previous manual methods, where measurement is more subjective and based on scoring (between 0 and 4 for no staining to intensive staining) (Ilanthodi, 2012). Further, the 3D-image analysis reconfirmed the specific expression of the gonadotropin and BMP receptors to follicular somatic cells (granulosa and theca cells) during different stages of follicle development i.e. primordial, primary, secondary and Graafian, which opened new facets of involvement of BMP signalling in the follicle development. The lack of gonadotropin receptor expression in primordial follicles suggested that the early stage of follicle development is gonadotropin-independent. The outcome of this study clarifies the ambiguity of the involvement of gonadotropin in primordial follicle development (Flaws et al., 1997, Meredith et al., 1992).

The longevity of female fertility is highly dependent on the number and quality of primordial follicles, the repetitive process of ovulation after puberty and continuous process of follicle atresia leading to the depletion of fertility reserve (Williams and Erickson, 2008). Hence, as a



part of the present study and the second aim of this thesis the role of BMP signalling in primordial follicle formation, primordial follicle recruitment, the primary follicle survival and ovulation were investigated using an *in vivo* mouse model. Attenuation of BMPR-1B and BMP-4 action was achieved by passive immunisation, and the effects were examined in the presence or absence of eCG, as a source of exogenous gonadotropin. The estimated number of primordial and primary follicles was counted using computer based stereological technique (Chapter 4). The results of this particular study showed that the effect of immunisation on primordial follicle reserve and ovulation rate was stronger in case of BMPR-1B than that of BMP-4 immunisation/attenuation, suggesting a lesser role for BMP-4 than BMPR-1B, in primordial follicle formation (Lawson et al., 1999) and recruitment (Tanwar et al., 2008). The passive immunised mouse model worked as a transient elicitor model. However, the effect of immunisation against BMP system showed an extended effect for several weeks after immunisation, which is unlikely due to the direct inhibitory action of antibodies. The increase in ovulation rate in vaccinated mice mimics the well-characterised feature of *Booroola* sheep (Mulsant et al., 2001, Souza et al., 2002, Ruoss et al., 2009), which exhibit mutation in BMPR-1B (Wilson et al., 2001, Campbell et al., 2009). Together with the other results in mice, sheep, and human, this study confirmed that attenuation in BMP signalling can be an effective approach to increase female fertility reserve by promoting follicle development and ovulation. As discussed later, eCG also had an influence on BMPR-1B expression.

With this result, the next aim was to investigate the mechanism of interaction between the gonadotropins and the BMP signalling during various stages of follicle development and constitutes the third aim of the thesis. The method adopted for this study was *in situ* immunological visualization with immunofluorescence quantification. The effect of passive immunisation of BMPR-1B, a common a receptor for BMP-4, on BMPR-1B, FSHR and LH expression was demonstrated in different stages of follicle development. Significant increase in BMPR-1B, FSHR and LHR expression was observed in mice vaccinated against BMPR-1B with and without eCG administration.

The results of the current study suggested coordination between the actions of BMP and gonadotropin consistent with previous reports (Miyoshi et al., 2006, Chen et al., 2013). In addition to BMPR-1B controlling the initial stages of primordial follicle recruitment, this study suggested an indirect role of BMPR-1B in the later stages of folliculogenesis, such as

secondary and Graafian follicles, as evidenced by increased FSH and LH receptor expression, which lead to increased follicular somatic cell sensitivity towards gonadotropins. The study also revealed a wider spectrum of expression of BMPR-1B and LHR as compared to FSHR due to the observed localisation of BMPR-1B and LHR in both granulosa and theca cells, whereas FSHR expression was restricted to granulosa cells. These results have suggested that the action of BMPs extends beyond the early stages of follicle development. Hence, the follicle development was shown to be regulated by direct actions of BMP in early stages of development as well as by indirect actions through increased sensitivity of the granulosa cells and up-regulation of gonadotropin receptors. The increases in the follicular somatic cells sensitivity towards gonadotropin enhances cellular proliferation (Findlay and Drummond, 1999), reduce apoptotic activity (Zhang et al., 2013), luteinisation and increases in the steroidogenic activity (androgen, oestrogen and progesterone production)(Miyoshi et al., 2006, Wang and Roy, 2009), which ultimately leads to improve the reproductive capacity. Interestingly, the granulosa cells of mice treated with eCG administration showed an increase in BMPR-1B and FSHR but not LHR expression. This observation suggested that the role of eCG in regulating and enhancing the gonadotropin could involve BMPs. This observation suggested that the role of eCG in regulating and enhancing the gonadotropin action via their receptors are mediated by BMPs.

The attenuation of BMP-4 through vaccination, either in the presence or absence of administered eCG, had little effects on BMPR-1B, FSHR and LHR protein expression. This observation suggested that the interaction between BMPs and gonadotropin occurred at an intra-cellular level (e.g. through regulating SMAD or cAMP). This observation also provides further confirmation that gonadotropins have no direct/obvious effects on the early stages of follicle development. Meanwhile, several reports confirmed that BMP-4 is a key player in regulating follicle development; our observation does not contradict the fact the BMP-4 is essential for follicle development as reported by skinner (Nilsson and Skinner, 2003) but rather suggests that BMP-4 may be involved in activation of other mechanisms, possibly by affecting apoptosis and/or enhancing primordial germ cell formation from mesenchymal stem cells (Zhou et al., 2011). However, this possible explanation requires further study.

There may be a role for interaction with epidermal growth factor (EGF). An in vitro study demonstrated that pre-antral follicle development and steroidogenic activity in mice can be modulated by the action of EGF (Almahbobi et al., 1995). In addition, it has been reported

that EGF appeared to down regulate the FSHR expression in mice (Findlay and Drummond, 1999) and a separate study showed that BMP-4 has inhibitory effects on EGF production in mice (Chen et al., 2013). Therefore, it may be possible that the attenuation of BMP-4 causes the up regulation of EGF which could abolish the eCG effect on gonadotropin receptor expression.

Finally, the interaction between the BMPs and gonadotropins was investigated at the nuclear level. The relative expression at transcript levels for BMPR-1B, FSH and LH receptors were studied in mice immunised against BMPR-1B or BMP-4 with and without eCG administration. Although whole ovaries were used in the experiment, the expression of FSHR and LHR were restricted to granulosa and theca cells.

In addition to the protein level interaction between the BMPR-1B and gonadotropin receptors in the previous study, which was visualized by *in situ* immune-localisation, this particular study revealed increase in FSHR and LHR transcript levels specific to ovaries immunised against BMPR-1B. The elevation of transcript levels of FSHR and LHR was observed in case of ovaries administrated with eCG indicating the involvement of gonadotropin in overall follicle development. However, the previous studies confirm non-dependence of gonadotropin on the early stages of folliculogenesis at protein level.

## Chapter 8 : Conclusion

Based on the data generated in this thesis, we consider that the current study is significant for several reasons. Firstly, the current study highlights the necessity and feasibility of using computerised 3D image analysis to detect and quantify the presence and distribution of a small quantity of molecules in a complex structure such as the ovarian follicles.

Secondly, this study demonstrates that the role BMP signalling system is likely to be an important regulator of ovarian function, in particular the process of follicle development, depletion and ovulation. Furthermore, the attenuation of BMP signalling system is an effective approach to increase female fertility reserve while promoting follicle development, ovulation and eventually the overall fertility. Furthermore, this study also demonstrated unequivocally the interaction between BMPs signalling and gonadotropin regulates the follicle development and provides a further confirmation that the process is tightly controlled by the BMPs action, directly by regulation the early stages for follicle development and indirectly by enhancing the follicle cells sensitivity towards gonadotropin hormones which ultimately leads to enhances the reproductive capacity.

The outcomes of the current study enhance our understanding of the mechanisms involved in the interplay between BMPs signalling and gonadotropin, hence the regulation of the rate of primordial follicle recruitment and depletion. This will have a great potential application in controlling the onset of menopause, hence increasing female fertility. Additional research and investigations are required to further determine the role of FSH and LH in ovarian BMPR expression with respect to specific BMP ligands. The findings of this study gives additional insights for the development of future preventative and/or therapeutic approaches to overcome female infertility.

## Chapter 9 : References

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## Appendix

Conference: Reproductive biology society of Australia

Decision: Oral Presentation/2012

Session: SRB Orals - Folliculogenesis and Embryo development

The temporo-spacial expression of BMP, FSH and LH receptors during early follicular development. (#149)

**Sarmed Al-Samerria<sup>1</sup>, Jim J McFarlane<sup>2</sup>, Ghanim G Almahbobi<sup>1</sup>**

1. Curtin University, Bentley, WA, Australia
2. Physiology, University of New England, Armidale, NSW, Australia

In mammals the ovary contains a finite number of primordial follicles, which serve as a fertility reserve for the whole reproductive age. Continuous and high rate usage of these follicles will result in complete depletion, cessation of ovarian function and infertility. We have reported that bone morphogenetic proteins (BMPs) are involved in the formation and development of ovarian follicles in human<sup>1</sup> and sheep<sup>2</sup>. Specifically, it is commonly believed that the process of initial recruitment of primordial follicles to the pool of growing follicle is not gonadotropin- but BMP-dependent. However, this has not yet been unequivocally confirmed. The aim of this study is to elucidate the interplay between BMPs and gonadotropins, FSH and LH in early folliculogenesis by demonstrating the temporo-spacial protein expression of the BMP receptors (BMPR), FSHR and LHR in reserve primordial and growing follicles. Ovarian serial 10 µm cryosections were prepared for *in situ* immunofluorescence labelling of several follicular stages and subjected to microscopy examination and computer-based quantitative analysis. Cell membrane-bound FSHR, LHR and BMPR are found in granulosa cells of the follicles. While BMPR is expressed in all follicle stages, FSHR is absent in granulosa cells of primordial follicles and LHR is absent in granulosa cells of both primordial and primary follicles. Quantitative analysis based on the intensity of fluorescent signals shows that the expression of BMPR, FSHR and LHR significantly ( $p < 0.001$   $p < 0.0001$   $p < 0.0001$  respectively) increases with follicular development. We conclude that the initial recruitment of primordial follicles to primary stage is possibly BMP-dependent with no apparent direct input of gonadotropins. The interplay between BMPs and gonadotropins becomes eventually effective from primary follicles onward. The outcome is significant in understanding the role of BMP signalling pathway in the regulation of the rate of primordial follicle depletion. This will have a great potential application in controlling the onset of menopause hence increasing female fertility.

1. Regan et al. 2011, WCRB
2. Ruass et al., 2009, Reproduction, 138:689

Passive immunisation is a useful model to study the role of BMPs in the regulation of ovarian follicle development in mice (#240)

**Sarmed Al-Samerria<sup>1</sup>, Ibtisam I Al-Ali<sup>2</sup>, Jim J McFarlane<sup>2</sup>, Ghanim Almahbobi<sup>1</sup>**

- 1. Curtin University, Bentley, WA, Australia**
- 2. Physiology, University of New England, Armidale, NSW, Australia**

Ovarian fertility reserve, present in form of primordial follicles, which represent the longevity and quality of reproductive activity. Complete depletion of fertility reserve with age due to high rate of consumption will lead to the cessation of ovarian function in mammals and specifically menopause in women. The initial transition from primordial to primary follicle is the key limiting step of follicle development and consumption. Folliculogenesis is primarily a gonadotropin-dependent process in addition to the role of several other factors such as the bone morphogenetic proteins (BMPs). However, the regulation of initial follicle development is generally known as gonadotropin-independent but other conflicting reports indicate otherwise and this discrepancy needs to be unequivocally clarified. Using a computerised stereological analysis, the aim of this study was to investigate the role of BMPs in the regulation of primordial follicle depletion and rate of primary follicle survival in different ages of passively immunised mice against BMP receptor 1B (BMPRII) and BMP-4 with and without treatment with the gonadotropin PMSG. The mean total numbers of primordial follicles in immunised mice with anti-BMPRII and anti-BMP-4 were significantly ( $p < 0.0001$ ) increased with ratios up to 205% to the numbers present in control animals of the same age. Treatment with PMSG alone has no effect on the numbers of primordial follicles. However, PMSG treatment of immunised mice abolished the increase in numbers of primordial follicles resulted from immunisation. The mean total numbers of primary follicles in immunised mice were also significantly ( $p < 0.0001$ ) increased with ratios up to 175% to those present in control animals of the same age. In contrast to the case of primordial follicles, treatment with PMSG alone or as a supplement with antibodies significantly reduced the number of primary follicles to a minimum ratio of 59% of follicles in control animals. The rates of primary follicle survival were decreased after puberty with age and particularly significant in treated animals. However, there was no significant difference in the survival rates of primary follicles between the different treatment groups within a given age.

## The effect of impaired function of BMPs by passive immunisation on the protein expression of BMPR1B, FSHR and LHR in the mouse ovary

**Sarmed Al-Samerria<sup>1</sup>**, Ibtisam Al-Ali<sup>2</sup>, Suresh Kumar K.A. Athiappan Palanisamy<sup>2</sup>, Ghanim Almahbobi<sup>1</sup>, James R McFarlane<sup>2</sup>

1. School of Biomedical Sciences, Curtin University, Perth, WA, Australia

2. Centre for Bioactive Discovery in Health and Ageing, University of New England, Armidale, NSW, Australia

The number and quality of ovarian follicles are important in determining the longevity and integrity of female fertility. It is well known that the survival and development of these follicles including ovulation, which results in the release of viable oocytes ready for fertilization, are controlled primarily by the gonadotropins. However, there is increasing evidence which suggests that there are several other factors such as the bone morphogenetic proteins (BMPs) which co-regulate ovarian function along with gonadotropins. In our recent studies in an attempt to shed light on the mechanism of action of BMPs, we have created an *in vivo* mouse model with attenuated BMP signalling using passive immunisation against BMPR1B and BMP-4. The aim of this study was to investigate the localisation of BMP receptor 1B (BMPR1B), FSHR and LHR in the ovaries of mice treated with anti-BMPR1B, and anti-BMP-4 with and without exogenous gonadotropins (eCG). BMPR1B was expressed in all follicle stages, FSHR was detected in primary follicles onward and LHR was absent in primary follicles but appeared in later stages. Quantitative analysis based on the intensity of fluorescent signals showed that the expression of BMPR1B, FSHR and LHR significantly increased in the granulosa cells of the pre-ovulatory and secondary follicles in mice treated with anti-BMPR1B. Mice treated with anti-BMP-4 show that the expression of BMPR1B and FSHR but not LHR increased significantly in pre-ovulatory follicles only with no effects observed in any other stages. The pre-ovulatory follicles in mice treated with eCG showed increased BMPR1B and FSHR but not LHR expression. These results together with our previous reports in sheep and mice confirm that the attenuation of BMP signalling system can be an effective approach to sustain the development of growing follicles, ovulation and consequently overall female fertility

**P-333 Passive immunization against bone morphogenetic protein signaling system is an effective model to increase female fertility reserve**

S. Al-Samerria<sup>1</sup>, J. McFarlane<sup>2</sup>, and G. Almahbobi<sup>1</sup>

<sup>1</sup>*Curtin University, School of Biomedical Sciences, Bentley WA, Australia,*

<sup>2</sup>*University of New England, School of Science and Technology, Armidale NSW, Australia*

**Study question:** the regulation of initial follicle development is generally known as gonadotropin-independent but other conflicting reports indicate otherwise and this discrepancy needs to be unequivocally clarified.

**Summary answer:** the aim of this study was to investigate the role of BMPs in the maintenance of primordial follicle reserve and rate of primary follicle survival in different age groups of passively immunized mice against BMP receptor 1B (BMPRII) and BMP-4 with and without treatment with the gonadotropin PMSG.

**What is known already:** Folliculogenesis is primarily a gonadotropin-dependent process in addition to the role of several other factors such as the bone morphogenetic proteins (BMPs). The initial transition from primordial to primary follicle is the key limiting step of follicle development and consumption.

**Study design, size, duration:** Using Passive immunization to the BMPs and computerised stereological analysis to determine the total number of primordial and primary follicles in mice ovary.

**Participants/materials, setting, methods:** \* Passive immunization.

\* Stereology for follicular counting.

\* Statistical Analysis.

**Main results and the role of chance:** The mean total numbers of primordial follicles in immunized mice with anti-BMPRII and anti-BMP-4 were significantly ( $p < 0.0001$ ) increased with ratios up to 205% to the numbers present in control animals of the same age. Treatment with PMSG alone has no effect on the numbers of primordial follicles. However, PMSG treatment of immunized mice abolished the increase in numbers of primordial follicles resulted from immunization. The mean total numbers of primary follicles in immunized mice were also significantly ( $p < 0.0001$ ) increased with ratios up to 175% to those present in control animals of the same age. In contrast to the case of primordial follicles, treatment with PMSG alone or as a supplement with antibodies significantly reduced the number of primary follicles to a minimum ratio of 59% of follicles in control animals. The rates of primary follicle survival were decreased with age after puberty and particularly significant decrease in treated animals. However, there was no significant difference in the survival rates of primary follicles between the different treatment groups within a given age.

**Limitations, reason for caution:** N/A

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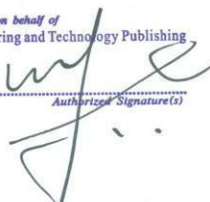
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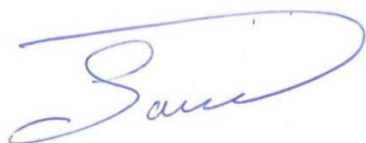
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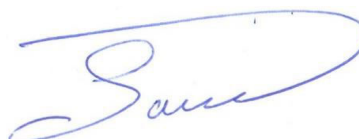
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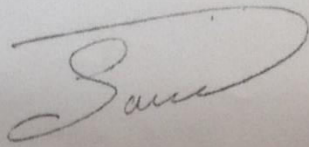
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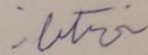


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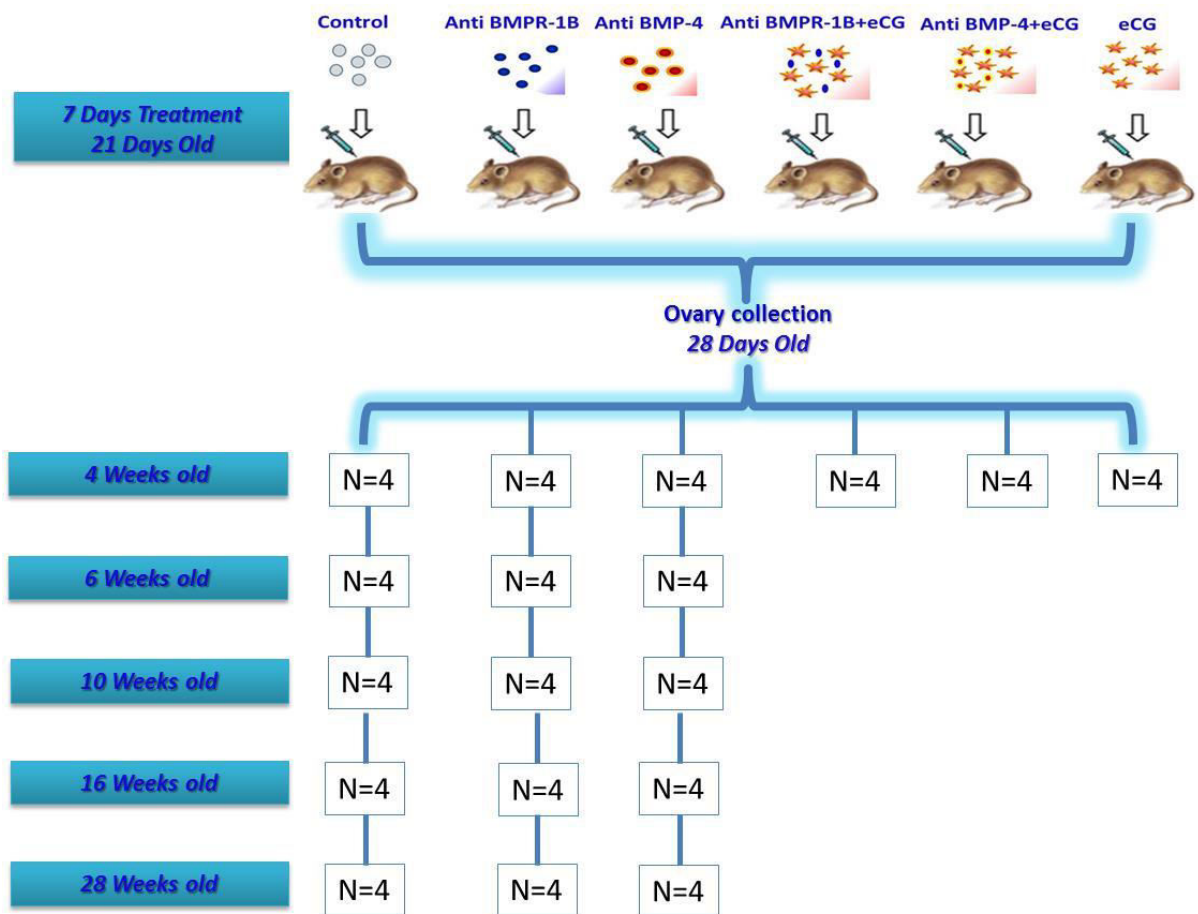
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**Figure 9-1: Animal groups and treatments**